The Osmotic Stress Response of Basidiomycetous Yeasts

By

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Declaration

I, **Obakeng McDonald Tekolo**, hereby declare that the work contained in this thesis is on my own original work and has not previously in its entirety or in part been submitted to any university for a degree.

Obakeng McDonald Tekolo: _____ Date: _____

Summary

Basidiomycetous yeasts are found in a wide range of geographical areas ranging from tropical forests to desert regions. These yeasts are associated with different habitats such as soil, decaying vegetative debris, living plants and animals. Some may even be opportunistic human pathogens. In most of these habitats the yeasts may periodically be exposed to adverse conditions such as osmotic stress. Forty-one basidiomycetous yeast strains obtained from culture collections and isolated from nature were studied using various methods which includes the determination of different minimum water activities (a_w; NaCl or sorbitol) for growth, survival in soil of varying moisture content, intracellular osmolytes accumulated and their release upon hypo-osmotic stress. The growth of most strains showed greater tolerance to NaCl than sorbitol at the same level of water activity. Interestingly, there were no basidiomycetous strains that showed growth below $0.90a_w$.

^{13}C nuclear magnetic resonance (NMR) spectroscopy and high performance liquid chromatography (HPLC) was used to analyze the osmolytes accumulated by all the strains of basidiomycetous yeasts when grown at 0.95 a_w (NaCl). Glycerol was the major solute accumulated intracellulary by all the yeasts. Arabitol, mannitol or trehalose was accumulated in addition to glycerol in most yeasts whereas a number of yeasts only accumulated glycerol when grown at reduced a_w. However, *Cryptococcus laurentii* US 1F was an exception by accumulating three solutes intracellularly when grown at reduced water activity. When exposed to hypo-osmotic shock all three solutes were rapidly released from the cells. *Cryptococcus hungaricus* CBS 5421, *Cryptococcus macerans* CBS 2206 and *Cryptococcus neoformans* US I1 were further analyzed with ^{13}C NMR spectroscopy to determine whether the type of osmolyte accumulated during different phases of growth at $0.95a_w$ (NaCl) might change. No changes were observed as the same osmolytes were accumulated in all cases.

Five yeast strains (C. *neoformans* US I1, *Rhodotorula mucillaginosa* CBS 5951, *C. macerans* CBS 2206T, *Filobasidium floriforme* CBS 6240 and *Sporidiobolus salmonicolor* CBS 5937) were analyzed by HPLC for osmolytes released when exposed to hypo-osmotic shock. The strains differed in the pattern of response of osmolyte release. Only three strains released most of their osmolytes rapidly within 5 min, while *C. macerans* CBS 2206T and *R. muculaginosa* CBS 5951 retained most of the

osmolytes intacellularly. This suggests that there might be different mechanisms of osmolyte release in basidiomycetous yeasts.

A few strains of basidiomycetous yeasts (*C. neoformans* US I1, *R. mucilaginosa* CBS 5951, *C. laurentii* 1F, *C. macerans* CBS 2206T, *F. floriforme* CBS 6240, *C. neoformans* CBS 0132, *C. laurentii* CBS 0139, *S. salmonicolor* CBS 5937 and *Filobasidium capsuligenum* CBS 4381) were grown in soil cultures of different field capacity (100%, 25%, 10% and 5%) and evaluated for their survival in this environment. All the strains grew at 100% field capacity. Strains *R. mucilaginosa* CBS 5951, *F. floriforme* CBS 6240 and *F. capsuligeum* CBS 4381 also showed growth in soil at 25% field capacity. However, strains *C. neoformans* US I1, *C. laurentii* US 1F, *C. macerans* CBS 2206T and *C. laurentii* CBS 0139 did not grow at this moisture content but survived up until the end of the experimental period. At lower soil moisture content (5% and 10% field capacity), the yeast strains either showed survival or decreased viability towards the end of the experimental period. Strain *C. neoformans* US I1, *C. laurentii* US 1F, *C. macerans* CBS 2206T and *R. mucilaginosa* CBS 5951 and *F. floriforme* CBS 6240 showed survival at both 5% and 10% field capacity. However, strain *F. neoformans* CBS 0132, *C. laurentii* CBS 0139, *F. capsuligenum* CBS 4381 and *S. salmonicolar* CBS 5937 showed a decrease in viability after either 2 or 5 days of incubation. No relationship could be found between the type and number of intracellular osmolytes accumulated when exposed to osmotic stress ($0.95a_w$ NaCl) and the ability to grow and survive in soil with lower moisture content. Similarly, the ability of the yeasts to grow and survive in soil with lower moisture content did not correlate with their minimum a_w for growth in a liquid medium. It was speculated that other factors, such as the physico-chemical composition of the soil, may also play a role in the survival of a particular yeast species in soil. This study has shown that the responses of basidiomycetous yeasts to reduced a_w are physiologically similar to the ascomycetous yeasts. The types of osmolytes accumulated are similar but the basidiomycetous yeasts appear to be more sensitive to reduced a_w and they tolerate NaCl better than sorbitol whereas the ascomycetous yeasts tolerate high sugar environments better. This is in agreement with the environments where these yeasts are usually found.

Opsomming

Basidiomisete giste word aangetref in ‘n wye reeks geografiese areas, wat strek vanaf tropiese woude tot woestynstreke. Hierdie giste word geassosieer met verskillende habitatte soos grond, verrottende vegetatiewe reste, lewende plante en diere. Sommige mag selfs opportunistiese menslike patogene wees. By meeste van hierdie habitatte mag giste periodies blootgestel word aan moeilike toestande soos osmotiese stres. Een-en-veertig basidiomisete gisrasse, verkry vanaf kultuurversamelings en geisoleer vanuit die natuur, was bestudeer met verskeie metodes, waaronder die bepaling van verskillende minimum water aktiwiteite (a_w; NaCl of sorbitol) vir groei, droë massa bepalings, akkumulasie van intrasellulêre osmoliete, asook hul vrystelling met hipo-osmotiese stres. Meeste rasse het meer weerstand teen NaCl as sorbitol gehad by dieselfde vlak van wateraktiwiteit. Dit was interessant om op te let dat geen basidiomisete stamme groei onder $0.90a_w$ getoon het nie.

Beide ^{13}C kern magnetiese resonansie (KMR) spektroskopie en hoë uitset vloeistof chromatografie (HUVC) was gebruik om alle opgehoopde osmoliete te analiseer vir alle basidiomisete gisrasse tydens groei tot by 0.95 a_w (NaCl). Vir alle giste was gliserol die opgelosde stof wat die meeste intrasellulêr opgehoop het. Arabitol of mannitol of trehalose het saam met gliserol in meeste giste opgehoop, terwyl ‘n aantal giste slegs gliserol opgehoop het tydens groei by verlaagde a_w. *Cryptococcus laurentii* US 1F was daarenteen ‘n uitsondering deurdat dit drie opgelosde stowwe intrasellulêr versamel het tydens groei by verlaagde wateraktiwiteit. Al drie hierdie opgelosde stowwe is uit die selle vrygesel na blootstelling aan hipo-osmotiese skok. Verdere ^{13}C KMR spektroskopie analise was gedoen op *Cryptococcus hungaricus* CBS 5421, *Cryptococcus macerans* CBS 2206 en *Cryptococcus neoformans* US I1 om vas te stel of die tipe opgehoopde osmoliet tydens verskillende fases van groei by $0.95a_w$ (NaCl) mag verander. Geen veranderinge was egter waargeneem aangesien dieselfde osmoliete in alle gevalle opgehoop het.

Vyf gisrasse (*C. neoformans* US I1, *Rhodotorula mucillaginosa* CBS 5951, *C. macerans* CBS 2206T, *Filobasidium floriforme* CBS 6240 en *Sporidiobolus salmonicolor* CBS 5937) was geanaliseer deur HUVC vir osmolietvrystelling tydens blootstelling aan hipo-osmotiese skok. Die rasse het verskil in hul responspatroon van osmolietvrystelling. Slegs drie rasse het meeste van hul osmoliete vrygestel binne die eerste 5 minute, terwyl ander, *C. macerans* CBS 2206T en *R. muculaginosa* CBS 5951

meeste van hul osmoliete intrasellulêr teruggehou het. Dit dui daarop dat daar dalk verskillende meganismes van osmolietvrystelling in basidiomisete giste mag voorkom.

Etlike basidiomisete gisrasse (*C. neoformans* US I1, *R. mucilaginosa* CBS 5951, *C. laurentii* 1F, *C. macerans* CBS 2206T, *F floriforme* CBS 6240, *C. neoformans* CBS 0132, *C. laurentii* CBS 0139, (B) *S. salmonicolor* CBS 5937, *Filobasidium capsuligenum* CBS 4381) was opgegroei in grondkulture van verskillende veldkapasiteit (100%, 25%, 10% en 5%) en ondersoek vir hul oorlewing in hierdie omgewing. Al die rasse kon groei by ‘n 100% veldkapasiteit. Die rasse *R. mucilaginosa* CBS 5951, *F. floriforme* CBS 6240 en *F. capsuligeum* CBS 4381 kon ook groei in grond met 25% veldkapasiteit. Alhoewel rasse *C. neoformans* US I1, *C. laurentii* US 1F, *C. macerans* CBS 2206T en *C laurentii* CBS 0139 nie kon groei by hierdie voginhoud nie, het hulle nog steeds oorleef tot aan die einde van die eksperimentele tydperk. By verlaagde grond voginhoud (5% en 10% veldkapasiteit) het die gisrasse of oorleef of ‘n verlaagde lewensvatbaarheid openbaar teen die einde van die eksperimentele prosedure. Die rasse *C. neoformans* US I1, *C. laurentii* US 1F, *C. macerans* CBS 2206T and *R. mucilaginosa* CBS 5951 en *F. floriforme* CBS 6240 het oorleef by beide 5% en 10% veldkapasiteit. Die rasse *F. neoformans* CBS 0132, *C. laurentii* CBS 0139, *F. capsuligenum* CBS 4381 en *S. salmonicolar* CBS 5937 het egter ‘n verlaging in lewensvatbaarheid getoon na ‘n periode van of 2 of 5 dae inkubasie. Geen ooreenstemming kon gevind word tussen die tipe en aantal intrasellulêre opgehoopde osmoliete nie na blootstelling aan osmotiese druk (0.95a_w NaCl), asook hul vermoë om te groei en oorleef in grond met ‘n laer voginhoud. Terselfdertyd het die giste se vermoë om te groei en oorleef in grond met ‘n laer voginhoud nie ooreengestem met hul minimum a_w vir groei in vloeibare groeimedium nie. Ter spekulasie kan ander faktore, soos die fisio-chemiese samestelling van die grond ook ‘n rol speel in die oorlewing van ‘n spesifieke gisspesie in grond.

Hierdie studie het getoon dat basidiomisete giste se respons tot verlaagde a_w fisiologies dieselfde is as vir askomisete giste. Alhoewel die tipe versamelde osmoliete identies was, wil dit voorkom asof basidiomisete giste meer sensitief teenoor ‘n verlaagde a_w is, tesame met ‘n beter bestandheid teen NaCl as sorbitol, terwyl askomisete giste hoë suiker omgewings beter kan verdra. Dit is in ooreenstemming met die tipe omgewings waar hierdie giste gewoonlik voorkom.

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Contents

Commonly used abbreviations

a_w	Water activity
CBS	Central Bureau voor Schimmelcultures
DHAP	Dihydroxyacetone phosphate
D_2O	Deuterium oxide
GPD	Glycerol-3-phosphate dehydrogenase
GPP	Glycerol phosphate phosphatase
HOG	High osmolarity glycerol
HPLC	High performance liquid chromatography
MEA	Malt extract agar
MAPK	Mitogen-activated protein kinase
^{13}C NMR	Carbon 13 nuclear magnetic resonance spectroscopy
YNB	Yeast nitrogen base medium

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1. Introduction

Basidiomycetous fungi derive their name from a cell structure known as the basidium (Prescott et al., 2002), and they are one of the five fungal divisions of the kingdom Eumycota (Deacon, 1980). They are commonly found on plants, animal materials, dry fruits, flowers, terrestrial and marine environments and in low concentrations in open ocean (Fell, 1976, Hohmann, 2002a, Tamas and Hohmann, 2003). Some species may show filamentous growth on wood and form mycorrhizal associations with the roots of trees (Deacon, 1980). Other basidiomycetous fungi may exist as yeasts. Generally yeasts are classified according to their morphological characteristics, sexual reproduction, physiological activities, and biochemical features (Barnett et al., 2000). They are described as unicellular fungi and eukaryotic micro-organisms metabolizing sugars, polyols, alcohols, organic acids and amino acids as sources of carbon and energy (Tamas and Hohmann, 2003). Although most of the relevant experiments were conducted with ascomycetous yeasts, and not with basidiomycetous yeasts, it is known that growth of yeasts is limited by low water activity (Hohmann, 2002b). Most micro-organisms that grow in environments of low water activity have an interior of lower water activity as revealed by the positive turgor pressure of the cells (Brown, 1978).

Water activity (a_w) is the term developed by the pharmaceutical and food industries (Grant, 2004). The term water activity (a_w) is defined as the ratio of equilibrium water vapour pressure of a solute to the saturated vapour pressure of the solvent (Khalloufi et al., 2000), or simply the amount of water available to micro-organisms. In addition, a_w measures the effectiveness of water concentration in the substrate and depends mostly on the solute concentration (Wolter et al., 2000). The term is related to osmotic pressure, osmolarity and water potential but the use of each term is generally specific to the nature of scientific literature. For example, a_w is commonly used in the microbiological and food science literature whereas water potential is found in the botanical literature. These industries use a_w to determine the shelf life and the value of the product (Grant, 2004). The units of water activity range from 1 to 0 where 1 reflects pure water whereas 0 reflects the absence of available water. Most environments where yeasts are found have an a_w greater than 0.5 (van Eck et al., 1993). Due to external influences and the activity of yeasts, water activity of their surrounding environment can range widely and rapidly. In such an environment, two aspects need to be considered by yeasts which is 1) to survive during sudden changes of water activity and 2) acquisition of tolerance to low water activity that will allow them to proliferate (Hohmann, 2002b).

Aims of the study

The aims of the study were the following:

1. To investigate the growth of selected basidiomycetous yeasts at various water activities (a_w's), and to establish the minimum a_w for growth
2. To determine the osmolytes accumulated by selected basidiomycetous yeasts in response to hyperosmotic stress
3. To investigate the release of osmolytes from selected yeasts when exposed to hypo-osmotic stress.
4. To investigate the survival of selected basidiomycetous yeasts in soil cultures at different field capacity

2. Literature Review

2.1. Osmotic stress

Changes that occur in the concentration of molecules (known as the osmoticum), which are dissolved in the medium surrounding the cell, may result in alterations in water availability to the cell and thus may cause osmotic stress. The higher the osmoticum concentration, the higher the osmolarity, but the lower the water activity. Ultimately, increasing external osmolarity will cause hyperosmotic stress and lead to water outflow from the cell (Figure 2.1). A decrease in the external osmolarity (hypo-osmotic stress) will generally result in an inflow of water into the cell (Tamas and Hohmann, 2003).

Osmotic stress triggers a set of cellular responses that enables the yeast to adapt to the changes in the environment. For example, when *Saccharomyces cerevisiae* is exposed to osmotic stress glycerol accumulates in the cytoplasm as a response to counteract cell dehydration (Albertyn et al., 1994; Holst, et al., 2000). In most cases studied so far, high concentrations of polyhydric alcohols or related compounds are accumulated by fungi in response to osmotic stress (Brown, 1978). The compounds that yeast cells produce and accumulate are known as compatible solutes or osmoprotectants and their function is to increase the internal osmolarity after hyperosmotic shock. On the other hand, after hypo-osmotic shock cells need to release the compatible solutes to avoid excessive turgor pressure and cell burst (Hohmann, 2002a; Kayingo et al., 2001; Tamas and Hohmann, 2003).

The cell regulates this condition by adjusting its level of intracellular solutes by activating membrane transporters to allow the inflow or efflux of solutes and ions coupled to changes in the cell volume (Kayingo et al., 2001). For example, cell shrinkage occurs due to the intracellular dehydration and growth arrest will occur temporarily (Hohmann, 1997). Furthermore, water influx or efflux elicits alterations in the structure of the cell such as in the plasma membrane (Tamas and Hohmann, 2003).

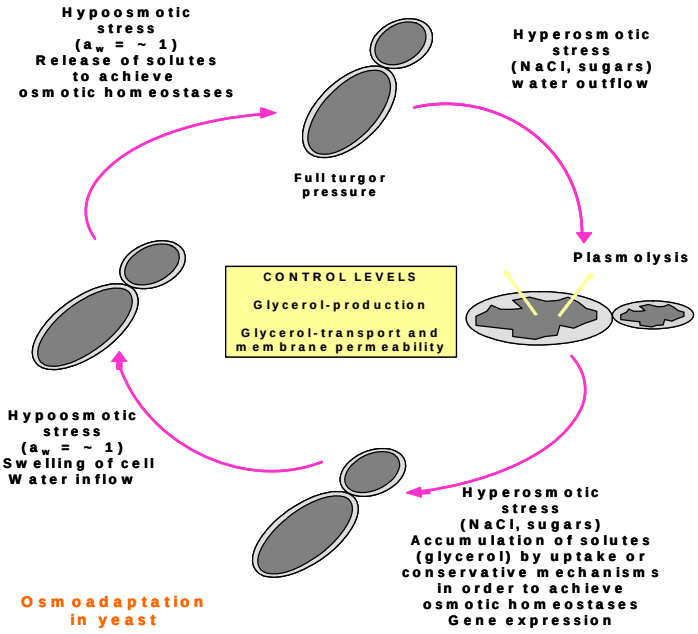

Figure 2.1: Schematic diagram of the response of *S. cerevisiae* in response to hyper- and hypo-osmotic shock (Tamas and Hohmann, 2003).

2.2. Sugar and salt tolerant yeasts

There are a variety of yeast genera that survive environments characterized by high sugar (Tokuoka, 1993). These microorganisms are termed osmophilic or osmotolerant. The term osmophilic is generally reserved for yeasts that require a significant sugar concentration for optimal growth, whereas osmotolerant yeasts such as *Pichia sorbitophila* are able to tolerate high sugar concentrations (40%-70%) but grow best in its absence. These yeasts normally occur in food where they have been found to cause spoilage. However, these yeasts may also play an important role in the production of fermented foods. The halotolerant microorganisms such as *Zygosaccharomyces rouxii* can tolerate both high sugar and salt concentrations during growth (Tokuoka, 1993).

The terms halophilic and halotolerant are applied similarly as osmophilic/osmotolerant but with reference to high salt concentrations. The salt-tolerant yeasts are specifically isolated from salty environments including foods such as soy source and miso paste. Yeasts that have been isolated from these foods include *Z. rouxii* and *Pichia anamola* (Spencer and Spencer, 1978). Much research has

focused on *Z. rouxii* as this yeast is a major cause of food spoilage. However, this species also plays an important role in producing foods such as soy sauce (Tokuoka, 1993).

2.2.1. The Osmotolerant yeasts species

Two genera, namely *Zygosaccharomyces* and *Schizosaccharomyces* (Spencer and Spencer, 1997) have been described as the major osmotolerant yeasts. The *Zygosaccharomyces* species include *Zygosaccharomyces bailii, Zygosaccharomyces bisporus, Zygosaccharomyces cidri, Zygosaccharomyces fermentati, Zygosaccharomyces florentinus,* and *Zygosaccharomyces mrakii,* whereas the *Schizosaccharomyces* species include *Schizosaccharomyces octosporus, Schizosaccharomyces japonicus, Schizosaccharomyces pombe* and *Schizosaccharomyces malidevorans*. Other osmotolerant yeast species such as *Hansenula anamola, Pichia farinosa, Debaryomyces hansenii, Candida torulopsi*, and *Candida magnoliae* have been reported (Spencer and Spencer, 1997).

2.3. Osmotic stress responses in yeasts:

2.3.1. Accumulation of compatible solutes in yeasts

Compatible solutes are defined as the replacement of cellular water by solutes without the normal functioning of the cell being impaired (Brown, 1974). These compatible solutes are described as osmoprotectants or osmolytes and include (i) ions, (ii) amino acids and (iii) polyhydroxy alcohols (Yancey et al., 1982). These osmolytes increase the osmolarity in the cell and this enables the cell to retrieve water from the environment in a competent way. Most microorganisms tend to use only one compatible solute, but some others use more than one. For example, *S. cerevisiae* produces only glycerol and may also produce glycogen and trehalose when under different levels of stress. However, yeasts predominantly use only polyhydroxy alcohols also known as polyols (Tamas and Hohmann, 2003).

2.3.2. Polyols produced in the cell

Yeast excretes polyols into their immediate surroundings when subjected to osmotic fluctuation (Tamas and Hohmann, 2003). These polyols include glycerol, mannitol, erythritol, arabitol and xylitol. The production of these polyols is also influenced by growth conditions or medium composition and the carbon source. For example, xylitol, is produced when xylose is used as a carbon source while *P. farinosa* produces glycerol and arabitol when grown on glucose, and mannose as carbon sources (Spencer and Spencer, 1978). However, a large number of yeast species produce glycerol intracellularly as the main osmolyte in response to hyperosmotic stress (Ansell et al., 1997). For example, *S. cerevisiae* and *Z. rouxii* produce glycerol during hyperosmotic stress and also excrete glycerol rapidly upon a decrease in external osmolarity (Luyten et al., 1995; Tamas et al., 1999; Kayingo et al., 2001). In addition, the importance of glycerol synthesis is to maintain the redox potential and consumptions of reducing equivalents in the cell (Ansell et al., 1997). However, yeast cells can also utilize glycerol as a source of carbon and energy in the presence of oxygen (Tamas and Hohmann, 2003).

2.3.3. Role of polyols in protection against osmotic stress

Polyols are produced intracellularly in order to counteract dehydration (Tamas and Hohmann, 2003). Apart from serving as compatible solutes, polyols are able to act as (i) carbohydrates and food reserves (Brown, 1978) (ii) translocatory compounds and (iii) function in coenzyme regulation and storage of reducing power (Jennings, 1984). In addition, these polyols also function to maintain osmotic equilibrium, stabilize and protect enzymes at low a_w activity without interfering with cellular metabolism (Brown, 1978). Polyols are normally required when the cells are exposed to a low a_w activity value. However, this low a_w activity causes yeast to regulate one or more solutes in order to adapt to the environmental changes although glycerol appears to be the major solute accumulated by most yeasts studied so far (Brown, 1978; van Eck et al., 1993).

2.3.4. The importance of water activity to growth

Water activity (a_w) is one of the major factors in the environment affecting the growth of micro-organisms (Tokuoka, 1993; Wolter et al., 2000). Water activity (a_w) can be considered or used to measure the water availability for yeast growth or survival (Hounsa et al., 1998; Wolter et al., 2000). Furthermore, yeasts have optimal and minimum a_w requirements (Graham, 2000). However, these microorganisms need to possess some mechanisms to avoid dehydration caused by osmosis when exposed to low a_w (Nobre and Da Costa 1993; Grant, 2004). Nevertheless, large numbers of yeasts are able to grow well at a_w around 0.95-0.90 (Deak, 2006). Despite this fact, most of fungi and yeast are inhibited at a_w values between 0.8 and 0.75 (van Eck et al., 1993; Grant, 2004). However, some yeasts such as *Z. rouxii* grow slowly at 0.62 a_w, in contrast to most bacteria, which are unable to achieve growth below 0.75 a_w (Wolter et al., 2000).

2.4. Polyol metabolism in *S. cerevisiae*

2.4.1. Glycerol metabolism

Glycerol is synthesized from dihydroxyacetone phosphate (DHAP), which may act as an intermediate of both glycolysis and gluconeogenesis (Figure 2.2) (Albertyn, et al., 1994; Ansell et al., 1997; Cronwright et al., 2002). Firstly, the dihydroxyacetone phosphate is reduced to glycerol-3-phosphate (G3P) catalyzed by NAD^+-dependent glycerol-3-phosphate dehydrogenase (GPD). Glycerol-3-phosphate is then dephosphorylated by NAD^+-dependent glycerol phosphate phosphatase (GPP) to form glycerol. These two enzymes are each encoded by two isogenes, namely, *GPD1* and *GPD2* in the case of GPD, while GPP is encoded by *GPP1* and *GPP2* (Albertyn, et al., 1994; Ansell et al., 1997; Eriksson et al., 1995). The expression of *GDP1* and *GPP2* are stimulated by high osmolarity (Albertyn, et al., 1994; Eriksson et al., 1995), while *GPD1* and *GPP2* are stimulated under anaerobic conditions (Ansell et al., 1997).

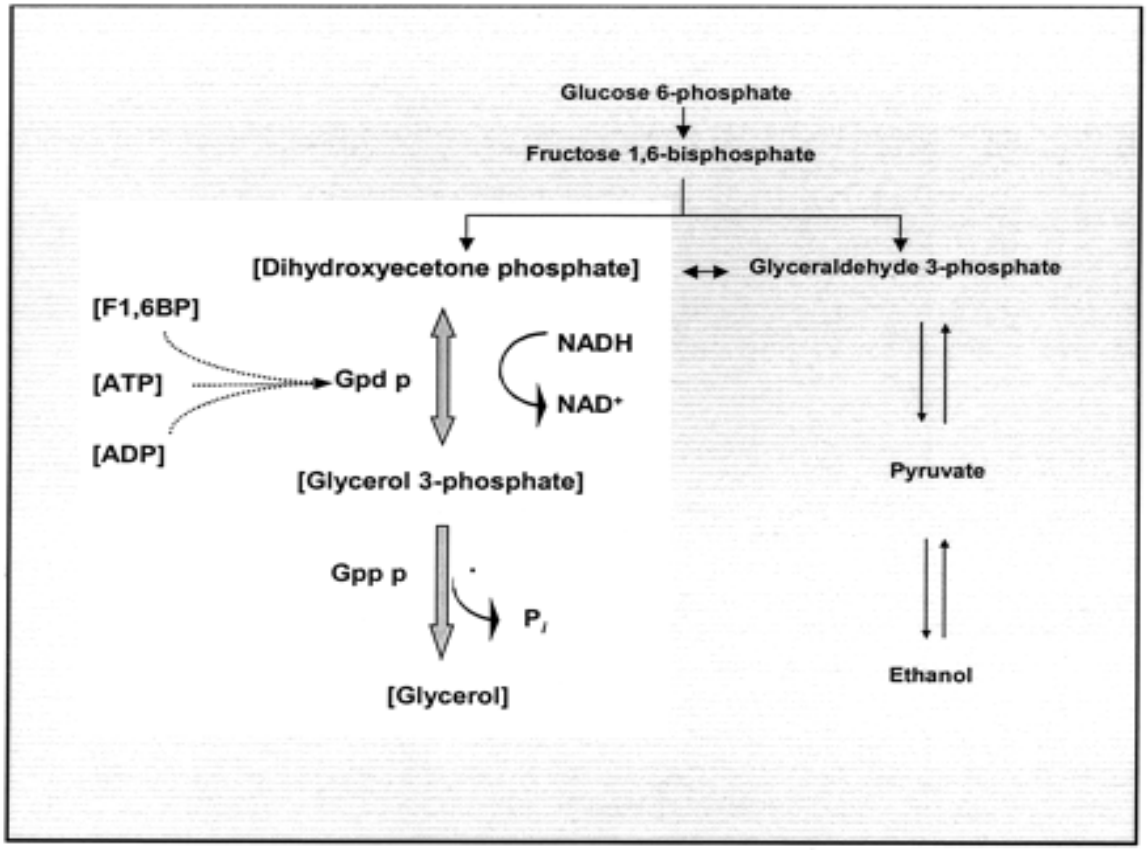

Figure 2.2: The formation of glycerol in *S. cerevisiae* (Cronwright et al., 2002)

2.5. Sensing of hyperosmotic stress: The Hog pathways

The HOG (high osmolarity glycerol) pathway is the major pathway in *S. cerevisiae* that is triggered by osmotic stress (Tang et al., 2005). As depicted in Figure 2.3 the HOG pathway consists of two transmembrane proteins, namely Sho1 and Sln1 (O'Rourke et al., 2002). These two transmembrane proteins are termed osmosensors that act on a downstream protein to regulate the MAPK Hog1. However, Hohmann (2002b) found indications that the Sho1 might not directly be sensing the osmolarity changes. Sln1 and Sho1 contain different functional domains and communicate directly to downstream effector proteins (O'Rourke et al., 2002). In low solute concentrations Hog1 phosphorylation depends on the Sln1 branch. However, in the presence of high solute levels the activity of the Sln1 branch is needed to stimulate the expression of several reporter genes (O'Rourke *et al*. 2002).

2.5.1. The Sln1 histidine kinase branch

Sln1, composed of two transmembrane regions and an intracellular histidine kinase domain, that function to provide signals to Ypd1 and Ssk1 proteins (O'Rourke et al., 2002). These two proteins and two transmembrane regions form a phosphorelay system (Figure 2.3). The phosphorelay system has a histidine kinase protein (Sln1) that transfers a phosphate group to an intermediate protein (Ypd1) which will transfer the phosphate to a response regulator protein (Ssk1). The Sln1 is active during constant osmotic conditions that lead to phosphorylation of the downstream target protein Ypd1 by transferring the phosphate group to the response regulator Ssk1. However, Ssk1 is also the phospho-acceptor in the phosphorelay system (O'Rourke et al., 2002). The Sln1p histidine kinase is activated by hypo-osmolarity and inhibited by hyperosmolarity. Ypd1p phosphorylates during the normal growth conditions, but after hyperosmotic shock phosphorylation is reduced (Hohmann, 2002b).

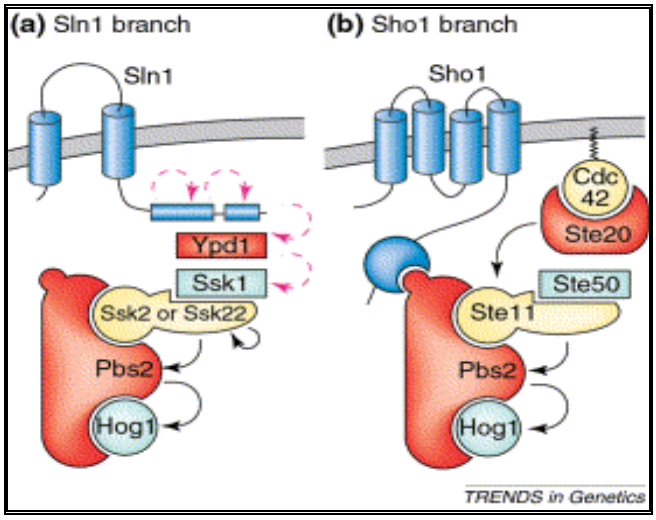

Figure 2.3: The schematic representation of the two osmosensors. Solid arrows indicate the phosphorylation events of threonine/tyrosine and serine during high osmolarity. The phosphotransfer events during stable osmolarity are indicated by the dashed arrows (O'Rourke et al., 2002).

2.5.2. The Sho1 branch

The Sho1 protein has four transmembrane segments and a C-terminal SH3 domain which interacts with downstream signaling elements in the HOG pathway (Hohmann. 2002b; O'Rourke et al., 2002). Recent studies have questioned the role of Sho1 as an osmosensor, hence, the activation mechanism of this branch of the pathway is not clear (O'Rourke et al., 2002). However, there are some indications that Sho1 might not directly sense osmolarity but may supply a docking site through its SH3 domain for downstream proteins. For example, when chimerical truncated variants of Sho1 protein are used for the stimulation of the HOG pathway, the transmembrane domains of Sho1 are not needed. The Sho1 branch uses SH3 domain encapsulated by a novel protein for monitoring the environment (O'Rourke and Herskowitz*,* 2004). In addition, Sho1 makes some use of several proteins, namely: Ste20, Ste50 and Ste11, these proteins are the components of two other mitogen-activated protein kinase (MAPK) cascades, the pheromone-response and invasive growth pathways (Figure 2.3).

2.6. Taxonomy of basidiomycetous yeasts

Basidiomycetes yeasts are described as unicellular fungi (Boekhout et al., 2000) that produce a yeast phase in which cells mostly multiply by budding (Carlile et al., 2001). Similar to most fungi the basidiomycetes are characterized by anamorphic (asexual) and teleomorphic (sexual) ontogenic stages in the life cycles (Gacser et al., 2001). These fungi are grouped into three classes (Figure 2.4), namely Hymenomycetes, Uredinomycetes and Ustilaginomycetes (Fell et al., 2000; Fell et al., 2001). Approximately 220 recognized species in 34 genera were assigned to these three classes using phylogenetic analyses based on 18S rDNA sequence analyses (Fell et al., 2001). The basidiomycetous yeasts were assigned to orders belonging to the classes mentioned above, namely Tremellales, Trichosporonales, Filobasidiales, Cystofilobasidiales, Ustilaginales and Sporidiales (Carlile et al., 2001; Fell and Scorzetti, 2004; Kurtzman and Fell, 2006). These orders were further grouped into families, such as the Cryptococcaceae and Sporobolomycetaceae (see 2.8.1).

Analyses of taxonomic informative gene sequences, within the ribosomal gene cluster, revealed the presence of four clades, representing the orders Tremellales, Trichosporinales, Filobasidiales and Cystofilobasidiales, within the subclass Tremellomycetidae of the Hymenomycetes (Gacser et al., 2001; Kurtzman and Fell, 2006). Interestingly, the genus *Cryptococcus*, which is a polyphyletic genus, was found to be present in all four clades (Gacser et al., 2001; Renker et al., 2004). The

Uredinomycetes, which are known to comprise of the rust fungi, was found to contain four major clades of yeasts and associated genera, namely *Agaracostilbales, Microbotryales, Sporidiobolales* and the *Naohidea* clade (Kurtzman and Fell, 2006). The Ustilaginomycetes, which mostly consists of plant and fungal parasites, are known to be associated with smuts (Kurtzman and Fell, 2006).

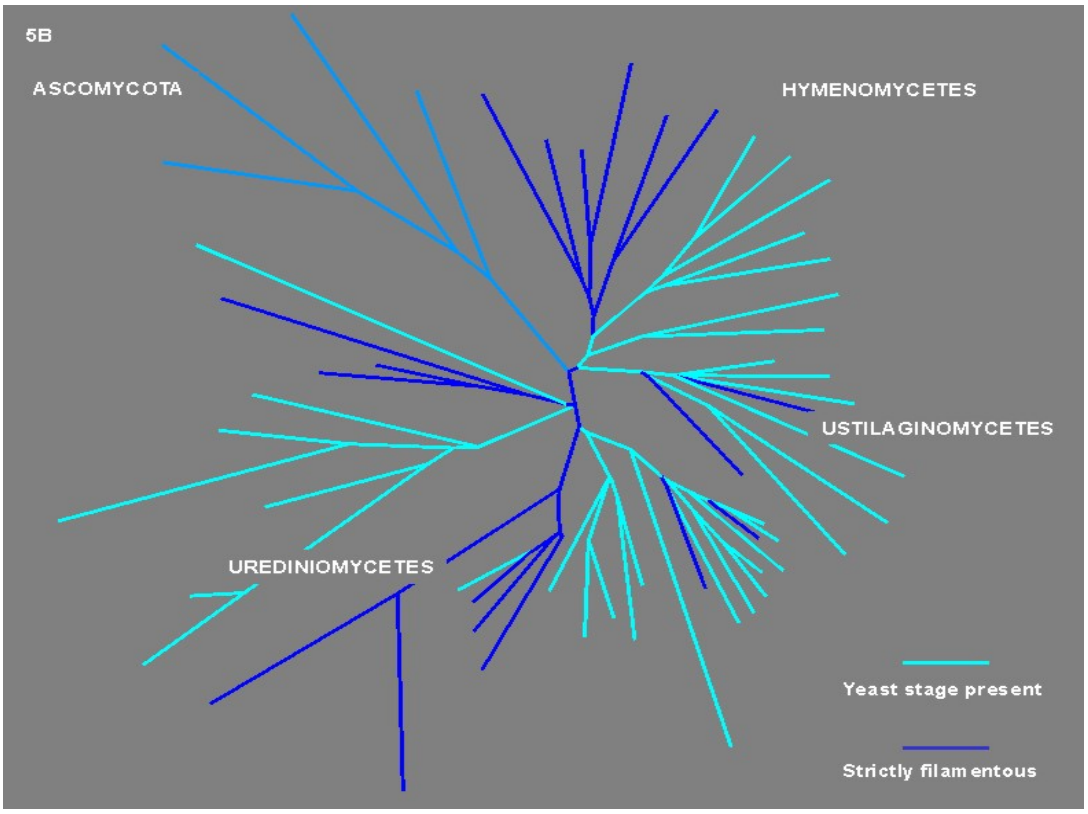

Figure 2.4: A phylogenetic tree constructed using taxonomic informative gene sequences, within the ribosomal gene cluster, of representatives of the Basidiomycota. It illustrates that the dimorphic basidiomycetes are extremely diverse and are distributed among three classes of the Basidiomycota: Urediniomycetes, Ustilaginomycetes and Hymenomycetes (Sampaio and Fonseca, 2002).

The best studied basidiomycetous yeasts, as a result of their ubiquitous nature and/or potential to act as pathogens, belong to the classes Hymenomycetes and Uredinomycetes. Examples of genera belonging to these classes are listed in Table 2.1. It can be seen that the polyphyletic genera *Cryptococcus, Sporobolomyces* and *Rhodotorula* are classified in more than one order (Fell et al., 2001; Kurtzman and Fell, 2006).

Species belonging to basidiomycetous yeast genera are identified using a combination of classical, morphological and physiological criteria as described by Kurtzman and Fell (2006), as well as molecular criteria based on taxonomic informative gene sequence. The latter include sequence comparisons of the internal transcribed spacer region (ITS) and large subunit D1/D2 region of the ribosomal gene cluster (Fell and Scorzetti, 2004).

Table 2.1. Classes and orders of basidiomycetous yeasts (Kurtzman and Fell, 2006)

[a]Hymenomycetes	[a]Uredinomycetes
[b]*Cystofilobasidiales*	[b]*Agaracostilbales*
Cystofilobasidium (T)	*Sterigmatomyces* (A)
Cryptococcus (A)	*Sporobolomyces* (A)
[b]*Filobasidiales*	[b]*Microbotryales*
Cryptococcus (A)	*Rhodotorula* (A)
Filobasidium (T)	*Sporobolomyces* (A)
	Rhodosporidium (T)
[b]*Trichosporonales*	[b]*Naohidea*
Cryptococcus (A)	*Rhodotorula* (A)
Trichosporon (A)	*Sporobolomyces* (A)
[b]*Tremellales*	[b]*Sporidiobolales*
Bullera (A)	*Sporidiobolus* (T)
Bulleromyces (T)	*Rhodosporidum* (T)
Cryptococcus (A)	*Rhodotorula* (A)

(A) = Anamorphic genus (T) = Teleomorphic genus

a = Class of basidiomycetous yeasts

b = Orders of basidiomycetous yeasts

2.7. Ecology of basidiomycetous yeasts

A well known habitat for basidiomycetous yeasts is soil (Botha, 2006). A large number of diverse basidiomycetous yeasts were found in soil from all over the globe. These yeasts were found in different geographical regions ranging from artic zones to the tropics (Atlas and Bartha, 1981; Botha, 2006). It is also known that some yeasts enter the soil thorough plant materials and are, therefore, known as allochnothonous yeasts. However, some of the basidiomycetous soil yeasts have been exclusively isolated from soil and are therefore called autochthonous soil yeasts. Species representing the genera *Rhodotorula* and *Cryptococcus* are the most abundant yeasts in soil. Examples of autochthonous

basidiomycetous soil yeasts include various *Cryptococcus* species, *Rhodotorula minuta* and *Sporobolomyces salmonicolor* (Spencer and Spencer, 1997). These yeasts are able to rapidly grow in soil under favourable conditions, such as in the presence of sufficient moisture and aeration, as well as in the presence of high concentrations of utilizable substrates (Atlas and Bartha, 1981). In many cases the unavailability of substrates in the soil causes fungi, including yeasts, to be dormant

As part of microbial biomass in soil, yeasts may play a role as a food source for bacteria and major soil predators such as micro-arthropods, nematodes, as well as protista (Botha, 2006). Furthermore, yeasts were found to be the nutrient source for a number of gram-positive and gram-negative soil bacteria. For example, myxobacteria were found to cause lysis of a broad diversity of yeasts, including *Cryptococcus albidus, Filobasidium capsuligenum, Rhodosporidium toruloides* and *Rhodotorula glutinis*.

Interestingly, while most autochthonous soil yeasts can metabolize simple carbohydrates, such as mono-saccharides, di-saccharides and tri-saccharides, none are known to effectively degrade complex polymers such as lignocellulose materials (Atlas and Bartha, 1981). However, a large number of yeasts from soil are capable of using aerobically the products of degraded wood material (cellulose and hemicellulose) such as L-arabinose, D-xylose and cellobiose and are also found to assimilate intermediates of lignin degradation (Botha, 2006). These soil yeasts form part of the degradation and energy flow process occurring in soil. Interestingly, yeasts may be the main culturable soil microbes found in some habitats, such as arctic zones. Under these conditions, these unicellular fungi play a significant role in the decomposition of the organic matter in the soil.

Another well known habitat of basidiomycetous yeasts is vegetative material, including living plants. A number of different yeasts occur on plants, especially aerial plant surfaces, and inhabit the leaves, flowers, stems and other parts of the plant (Spencer and Spencer, 1997; Atlas and Bartha, 1981). These yeasts are spread by insects and bees where the insects act as a carrier of the yeasts from plant to plant and bees carry yeasts from flowers to the honey reserves in the nests (Spencer and Spencer, 1997). Examples of basidiomycetous yeasts found on plant surfaces include *Sporobolomyces roseus, Rhodotorula glutinis, Rhodotorula mucilaginosa*, and *Cryptococcus laurentii* (Spencer and Spencer, 1997; Atlas and Bartha, 1981). The population of these yeasts on the leaves is influenced by the nutrients from leaf exudates and the decaying leaves. A factor that increases the population of these yeasts, is nitrogenous compounds produced by nitrogen-fixing bacteria harboured by tropical plants.

In addition, some bacteria are known to produce or transform sticky sugary materials that are metabolized by these yeasts, thereby having a positive impact on the populations of these unicellular fungi (Spencer and Spencer, 1997). Basidiomycetous yeasts are also found in marine waters, and the most frequently found isolates belong to the genera *Cryptococcus, Trichosporon, Rhodotorula and Rhodosporidium* (Atlas and Bartha, 1981).

2.8. Morphological features of basidiomycetous yeasts

2.8.1 Characteristics of orders and families

The basidiomycetes all produce characteristic sexual cycles that contain the spores on basidium or club like structure (Kurtzman and Fell, 2006). In the absence of characteristic sexual structures anamorphic basidiomycetous yeasts are identified by the manner in which budding takes place. However, cell fission and the formation of ballistoconidia may also occur among these yeasts (Fell et al., 2001).

2.8.2. The anamorphic basidiomycetous yeasts

The term anamorphic yeast refers to yeasts with no known sexual cycle (Carlile et al., 2001; Spencer and Spencer, 1997). Spores are asexually produced and budding occurs enteroblastic, while cell walls are lamellate. Hyphal septa may contain dolipores if perforated, but parenthesomes in septa may be formed by some species.

The anamorphic basidiomycetous yeasts are assigned to two polyphyletic families namely, Cryptococcaceae and Sporobolomycetaceae. The genera assigned to these families are listed in Table 2.1 and included, C*ryptococcus*, *Rhodotorula* and *Sporobolomyces.* In the past, the descriptions of anamorphic genera were based on phenotypic characteristics. For example, the genus *Rhodotorula* was known to be characterized by pink pigmented colonies. However, after sequence analyses of taxonomic informative gene sequences within the ribosomal gene cluster, basidiomycetous yeast species with white and cream coloured colonies were also included in the genus *Rhodotorula* (Kurtzman and Fell, 2006).

2.8.2.1. Sporobolomycetaceae family

Typical of basidiomycetous yeasts, budding in this family occurs enteroblastic and cell walls are lamellate. Hyphal septa are diaphragm-like. The main genus of the Sporoblomycetaceae is *Sporobolomyces* while the other genera include *Rhodotorula* and *Sterigmatomyces* (Boekhout et al., 2000). Representatives of *Sporobolomyce*s multiply by budding but may also produce a single ballistospore on a sterigma. However, most anamorphic yeasts do not form ballistospores (Carlile *et al*., 2001). The genus *Rhodotorula* is characterized by pink pigmented colonies and budding cells with narrow bud scars. *Sporobolomyces,* which is the anamorph of *Sporidiobolus,* also produces pink colonies, but may be differentiated from *Rhodotorula* species (Guarro et al., 2000) by the presence of ballistoconidia (Fell et al., 2001).

2.8.2.2. Cryptococcaceae family

The family Cryptococcaceae is restricted to anamorphic heterobasidiomycetous yeasts and yeast-like fungi (Boekhout et al., 2000). The main genus of this family is *Cryptococcus*, but other genera include *Bullera, Trichosporon* and *Fellomyces* (Boekhout et al., 2000).

2.8.3. Teleomorphic basidiomycetous yeasts

Teleomorphic basidiomycetous yeasts are characterized by morphological features that include the presence of ballistoconidia, dikaryotic hyphae, clamp connections, teliospores and basidia (Fell et al., 2001). However, the Basidiomycetes with yeast and yeast-like states are differentiated by basidial morphology, septal spore anatomy and cell wall composition. The classification of families and orders rely mostly on basidial morphology (Boekhout et al., 2000).

2.8.3.1. Sporidiobolaceae family

The family of Sporidiobolaceae is restricted to teliomorphic yeast and yeast-like fungi and is included in the order Sporidiobolales, characterized by enteroblastic budding and thick-walled teleospores. The mycelium of this family is dikaryotic, with or without clamp connections, and it forms no haustorial branches. The basidia are single celled, while the cell walls are lamellate, and hyphal septa have attenuated diaphragm-like septa if perforation takes place. In this case perforation takes place without

the presence of woronin-bodies and dolipores (Boekhout et al., 2000). The main genus of this family is *Sporidiobolus.* Other genera include *Leucosporidium* and *Rhodosporidium* (Boekhout et al., 2000). An example of a teleomorphic basidiomycetous yeast, producing both asexual and sexual ontogenic stages, is that of *Rhodosporidium toruloides* (Figure 2.5).

2.9. The life cycle of a basidiomycetous yeast

Rhodosporidium toruloides is a typical basidiomyceous yeast, which may be homothallic or heterothallic (Fell and Statzell-Tallman, 2000). In heterothallic systems a dikaryotic mycelium develops from a compatible mating pair i.e. mating types A1 and A2 (Figure 2.5). The mycelium comprises of septate hyphae with clamp connections at the septa. The function of these clamp connections is to ensure an even distribution in the hyphae of the daughter nuclei originating from the two mating types.

A clamp connection within a dikaryotic hypha is formed during cell fission when a hyphal outgrowth is formed opposite to the direction of growth of the hyphal tip (Figure 2.6). One nucleus of the dikaryon then divides along the main axis of the hypha, while the other divides into the clamp (Swann, 1997). A septum then forms within the original apical cell resulting in an apical dikaryon, containing a daughter nucleus originating from each mating type, and a sub-terminal cell containing only one nucleus. The hyphal outgrowth, containing one of the daughter nuclei, subsequently fuses with the sub-terminal cell allowing the nucleus to migrate into this cell. Consequently, the dikaryon is reestablished in the sub-terminal cell.

After a few weeks of growth thick-walled teliospores are formed terminally or laterally on clamped sporophores in the dikaryotic mycelium (Fell and Statzell-Tallman, 2000). Karyogamy occurs within these irregular shaped spores (Figure 2.5) which are known to survive adverse conditions. Within one or two weeks meiosis occurs and the teliospores may germinate giving rise to club shaped metabasidia containing at least 2-4 cells each. The budding of uninucleate basidiospores develop laterally and terminally. Some of these basidiospores are heterothalic and are able to mate and repeat the life cycle.

Homothallic basidiospores produced on the metabasidium, however, develop directly into a dikaryotic mycelium with clamp connections and teliospores (Fell and Statzell-Tallman, 2000). The resulting

teliospores may germinate again to generate or produce metabasidia with heterothallic and homothallic basidiospores.

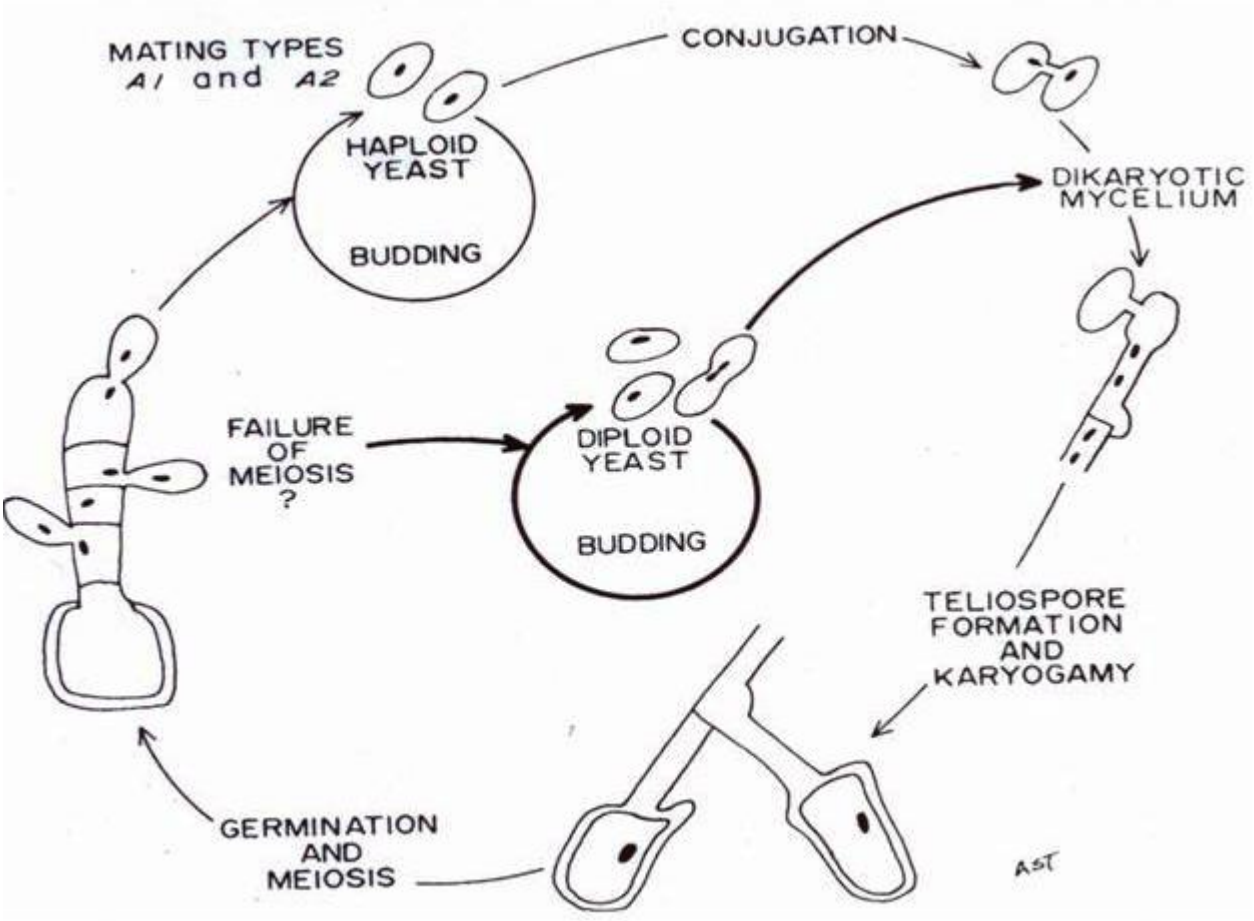

Figure 2.5: The schematic representation of the general life cycle of *Rhodosporidium toruloides* (Fell and Statzell-Tallman, 2000).

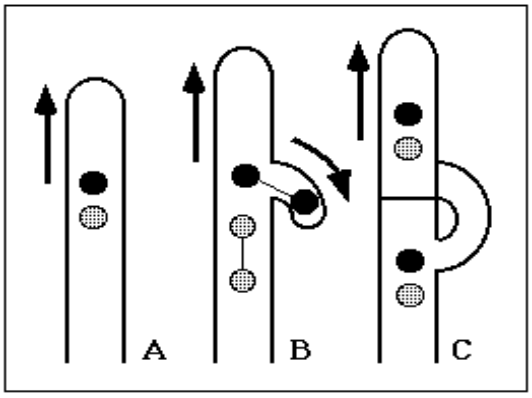

Figure 2.6: Schematic representation of the division of dikaryotic hyphae forming clamp connection (Swann, 1997).

2.10. The medical importance of basidiomycetous yeasts

The prevalence of immunocompromised patients and the greater usage of antibacterial antibiotics have resulted in the basidiomycetous yeasts increasing in importance as a medical problem (Fell et al., 2001). Most of the genera with pathogenic characteristics are classified into the order Tremellales and in the genera *Cryptococcus, Trichosporon,* and *Moniliella.* Basidiomycetous yeast species known to be potentially pathogenic include *Filobasidiella neoformans,* which is described as the sexual phase of *Cryptococcus neoformans* (Carlile, 2001), *R. glutinis, R. mucilaginosa* and *Rhodotorula minuta* (Guarro et al., 2000).

The most important genus from a medical point of view is *Cryptococcus* (Guarro et al., 1999). *Cryptococcus* is a large genus with 34 species of diverse physiological relationships (Guarro et al., 1999). The two major pathogenic species in the genus are *C. neoformans* and *C. laurentii. Cryptococcus neoformans* is an encapsulated opportunistic pathogen (Figure 2.7), which causes cryptococcosis by the inhalation of the airborne cells, and causes lungs, respiratory and neurological disease in animals and humans (Sorrel and Ellis, 1997; Carlile et al., 2001). The yeast is found in both immunocompetent and immunocompromised individuals. In most immunocompetent individuals, the infection remains dormant until an imbalance of the immunity occurs. However, in case of immunocompromised patients, the fungus spreads to different organs causing cryptococcal infection that leads to lung infection or pneumococcal-type pneumonia in the lungs and meningitis or meningoencephalitis in the central nervous system (Buchanan and Murphy, 1998; Cogliati et al., 2000; Carlile et al., 2001; Fell et al., 2001; Bose et al., 2003). The yeast is particularly virulent in AIDS and tuberculosis patients (Carlile et al., 2001).

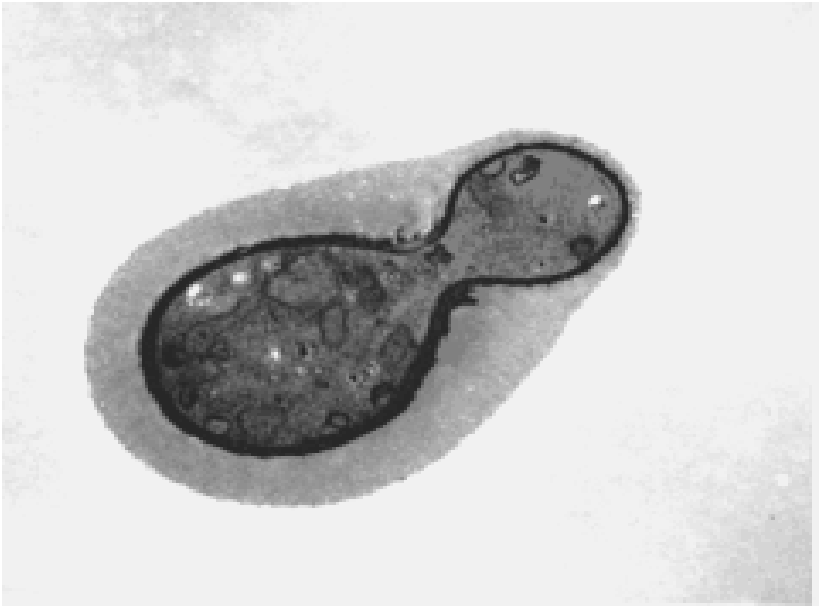

Figure 2.7: Electron micrograph of budding *C. neoformans* enclosed in a capsule (Buchanan and Murphy, 1998)

Another genus harbouring potentially pathogenic yeasts is *Trichosporon.* This genus is characterized by the formation of true hyphae which disarticulate to produce arthroconidia during asexual reproduction (Middelhoven et al., 2001). Members of this genus were found to cause respiration and renal failure, as well as intravascular coagulation syndrome in immunodeficiency patients (Diaz and Fell, 2004). *Trichosporon cutaneum Trichosporon mucoides* and *Trichosporon ovoides* cause the disease known as Trichosporonosis which is reported to cause mortality in immunocompromised patients (Sugita et al., 1998; Diaz and Fell, 2004). *Trichosporon* species have been isolated from different environments including soil, animals and man (Guarro et al., 1999)

3. Materials and Methods

3.1. Strains

Forty one strains (Table 3.1) of basidiomycetous yeast representing 28 species were used in this study. Ten strains were provided by the Microbiology Department, University of Stellenbosch (US) and 31 strains were obtained from the University of the Free State. These cultures originated from either the Council for Scientific and Industrial Research (CSIR), Pretoria or the Centraal Bureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

3.2. Growth conditions

The strains were maintained at 4^{o}C, on yeast malt extract (YM) agar containing (per litre of distilled water) 3.0g malt extract (Merck), 3.0g yeast extract (Merck), 5.0g meat peptone (Merck), 10.0g D-glucose (Merck) and 20.0g agar (Merck). The yeast cultures were sub-cultured on YM agar every three months, allowed to grow for 72 hours at 25^{o}C, and stored at 4^{o}C. All the growth experiments were conducted in yeast nitrogen base medium supplemented with amino acids (YNB; 7.5g /l; Difco) and D-glucose (100g/l).

3.3. Determination of minimum a_w for growth

The yeast culture was inoculated into 50 ml (250 ml Erlenmeyer flask) of glucose-YNB medium and incubated by vigorous agitation on a rotary shaker (180 rev/ min) at 30^{o} C until the exponential growth stage. The inocula were seeded into 4.5 ml glucose-YNB adjusted to water activities (0.998, 0.975 0.95, 0.925, 0.90, and 875) with NaCl or sorbitol in 10 ml test tubes. The cultures were incubated at 30^{o}C and vortexed every 4 h with a Vortex mixer. A Genesys 20 spectrophotometer was used to monitor growth of organisms by measurement of absorbance at 600 nm. The readings were taken at intervals of 4 h for at least 60 hours.

Table 3.1: Basidiomycetous yeasts used in the present study

Species	Accession source and number
Bulleromyces albus	CBS 6097 (CSIR Y-498)
Bullera dendrophila	CBS 6074T (CSIR Y-499)
Cryptococcus albidus	CBS 5737 (CSIR Y-73)
Cryptococcus amylolentus	CBS 6039T (CSIR Y-501)
Cryptococcus bhutanensis	CBS 6294T
Cryptococcus curvatus	CBS 2176
Cryptococcus gastricus	CBS 1927
Cryptococcus hungaricus	CBS 5124 (CSIR Y-548)
Cryptococcus laurentii	US 1A
Cryptococcus laurentii	US 1F
Cryptococcus macerans	CBS 2206T
Cryptococcus neoformans	US 132T
Cryptococcus neoformans	US C2
Cryptococcus neoformans	US I1
Cryptococcus neoformans	US I5
Cryptococcus neoformans	US I6
Cryptococcus neoformans	US S5
Cryptococcus neoformans	US I4
Cryptococcus podzolicus	US 5A
Cryptococcus terreus	CBS 1895T
Cryptococcus laurentii	CBS 0139
Filobasidium capsuligenum	CBS 4381
Filobasidium capsuligenum	CBS 6122.2
Filobasidium floriforme	CBS 6240
Filobasidiella neoformans var *neoformans*	CBS 0132
Filobasidiella neoformans var *neoformans*	CBS 0884
Filobasidiella neoformans	CBS 6885
Filobasidium unigetulattum	CBS 2770
Rhodotorula araucariae	CBS 6031T
Rhodotorula glutinis	CBS 0020
Rhodotorula graminis	CBS 2826
Rhodotorula lactosa	CBS 5826T
Rhodotorula minuta var *minuta*	CBS 2172
Rhodotorula minuta var *minuta*	CBS 2177
Rhodotorula mucilaginosa	CBS 5951
Rhodosporidium toruloides	CBS 0349
Rhodotorula sp	CBS 5143 (UF-448)
Sterigmatomyces halophilus var *halophilus*	CBS 4609Y (N-4619T)
Sterigmatomyces halophilus	CBS 5628Y (N-6837))
Sporidiobolus salmonicolor	CBS 5937Y (CSIR Y-144)
Trichosporon cuteneum var *cuteneum*	CBS 2644NT (CSIR Y-351)

3.4. Hypo-osmotic shock and efflux experiments

The yeast culture was inoculated into 100 ml (250 ml Erlenmeyer flask) of glucose-YNB medium and incubated by vigorous agitation on a rotary shaker (180 rev/ min) at 30^{o}C until the exponential phase. The cells were harvested by centrifugation (5,000xg, 5min) and resuspended in 100 ml of glucose-YNB containing 5 % NaCl (0.972a_w) and grown by vigorous agitation on a rotary shaker (180 rev/ min) at 30^{o}C for 3 h. The hypo-osmotic shock was performed by diluting the cell suspension ten-fold with glucose-YNB medium (0.998 a_w) medium at 30^{o}C. After the given time interval, cells were instantly sedimented and the pellets were saved for intracellular osmolyte determination.

3.5. Extraction and measurements of osmolytes

The extracted cells were washed twice with cold distilled water (5,000xg, 5min), resuspended in distilled water, boiled at 100^{o} C for 10 min and centrifuged (5,000xg, 5min) to remove the debris. The supernatant was retained for the analysis of intracellular osmolytes (glycerol, arabitol, mannitol and trehalose). The osmolytes were separated on a MA1 column at a flow rate of 0.25 ml/min with double distilled water and 500 mM of NaOH as eluent. Osmolyte concentrations were determined by high performance liquid chromatography (HPLC, Dionex) with an electrochemical detector. All the samples were filtered through 0.22 μm filters (Kimix) before separation and analyzed with HPLC.

3.6. Dry mass measurements

Cultures were grown to an exponential phase and diluted with sterile glucose-YNB or glucose-YNB with NaCl (0.972 a_w). The turbidity was measured with a Genesys 20 spectrophotometer at 600nm with glucose-YNB medium as a blank. Pre-weighed membrane filters (0.45 μm) were used for harvesting the cells (10 ml) under vacuum and washed with an equal volume of distilled water. Filters with cells were oven-dried at 80^{o}C for 24 hours and left in a desiccator overnight. The weight of the membrane filters was measured and the difference from the initial weight was calculated as the dry mass/ 10ml. A standard curve relating absorbance to biomass concentrations was constructed.

3.7. Identification of accumulated polyols by natural abundance ^{13}C NMR spectroscopy

The yeast culture was grown to exponential phase in 100 ml (250 ml Erlenmeyer flask) of glucose-YNB medium adjusted with 0.95 a_w NaCl (7.2% NaCl) or 0.998 a_w (0% NaCl), and incubated with vigorous agitation on a rotary shaker (180 rev/ min) at 30^o C. The cells were harvested by centrifugation at 15,000xg for 10 min, washed once with sterile water and the cell pellet was placed in a 2 ml Eppendorf tube for ^{13}C nuclear magnetic resonance (NMR) spectroscopy analysis. Approximately 600 µl of D_2O (deuterium oxide) was added to the tube to aid in the analysis of the samples with ^{13}C NMR spectroscopy and mixed. ^{13}C NMR spectra were collected on a Varian ^{Unity}*Inova* 600 spectrometer (operating at 150 MHz for ^{13}C) using a 5 mm broad band probe. Samples were run at 20 ^{0}C with a 2.5 sec acquisition, 1 sec pulse delay, approximately 60^0 pulse angle and inverse gated decoupling. Spectra were accumulated until sufficient signal to noise was obtained for analysis of the data (1000 - 17000 scans). Dioxane was used as an external reference standard.

3.8. Effect of stage of growth on intracellular solute accumulation as identified by ^{13}C NMR spectroscopy and HPLC

The yeast culture was grown to exponential phase in 100 ml (250 ml Erlenmeyer flask) of glucose-YNB medium adjusted to 0.95 a_w with NaCl (7.2% NaCl) or 0.998 a_w (0% NaCl), and incubated with vigorous agitation on a rotary shaker (180 rev/ min) at 30^o C. The cells were harvested after either 4 h or 6 h intervals by centrifugation at 15,000xg for 10 min, washed once with sterile water and the cell pellets were placed in 2 ml Eppendorf tubes for ^{13}C-nuclear magnetic resonance (NMR) spectroscopy analysis. Analysis was conducted as described in 3.7.

3.9. Assay of viability after hypo-osmotic shock

Yeast cultures were grown to exponential phase and harvested by centrifugation (5,000xg, 5 min), resuspended in YEPD medium (1 % yeast extract, 2 % peptone, 2 % glucose) adjusted to 0.972 a_w (5% NaCl) and incubated for 3 h. Cells under stress were harvested (5,000xg, 5 min), washed twice with the sterile YEPD medium and resuspended in YEPD medium with 0.972 a_w. The cell suspension was diluted serially in 1ml YEPD with or without NaCl. Samples (0.2 ml), taken from the 10^{-6} dilutions, were plated on the YEPD media containing the same concentration of NaCl as the diluting medium. The plates were incubated at 30^o C until the colonies were visible.

3.10. Survival of selected yeasts in soil differing in moisture content

3.10.1. Preparation of soil substrate containing woody debris.

An *Acacia mearnsii* tree from the Stellenbosch area was selected and felled. The tree was split into four components, namely the main stem, branches, twigs and leaves. The main stem was chipped with a Wigger pilot-size chipper, and a randomized chip sample was taken. The sampled stem material consisting of relative large wood chips, as well as the branch, twig and leave components, was dried for 24 h at 100º C and put through a Condux-Hype type hammer mill giving smaller chips, resembling the size of match-sticks. The smaller-sized chips of all four tree components were sub-sampled and each sample was further reduced in size, with a Retsch (ZM-1) ultra centrifugal mill with a 6 mm sieve, resulting in roughly the same particle size distribution for each of the four components. The four components were subsequently mixed in a ratio of 50 % (w/w) main stem, 16 % (w/w) branches, 16 % (w/w) twigs and 16 % (w/w) leaves.

Red clay (Corobrick Brick Works, Stellenbosch) was dried at 100º C for 48 h, ground using a pestle and mortar and sieved using a 2.0 mm sieve. The woody debris mentioned above was mixed in a 50/50 % (w/w) ratio with the clay to obtain the soil used in this study.

3.10.2. Determination of field capacity

A column containing the soil mix mentioned above was saturated with approximately 300 ml water and allowed to dry by gravitation for 48 h. The soil was removed, weighed and dried at 110° C for 24 h after which the dried substrate was weighed. The field capacity was calculated as a percentage (w/v) with regards to the amount of water absorbed and then lost by the substrate.

3.10.3. Preparation of soil cultures

Selected yeast cultures were each inoculated into 25 ml of YM medium containing (per litre of distilled water) 3.0 g malt extract (Merck), 3.0 g yeast extract (Merck), 5.0 g meat peptone (Merck) and 10.0 g D-glucose (Merck) in 250 ml Erlenmeyer flasks. The flasks were incubated at 26^0 C for 48 h on a rotary shaker (120 rev/ min). The cultures were harvested with centrifugation (12 000 x g, 10 min) and washed twice with distilled water. The concentration of yeast in the suspension was determined using a

heamocytometer under a light microscope. In each case an appropriate dilution was prepared containing a predetermined yeast concentration that was used as inoculum for a soil culture. Soil cultures were prepared by inoculating each of a series of 50 ml glass bottles, each containing 10 g of soil (red clay and *Acacia* debris mixed in 50/50 %, w/w, ratio), with 4.5 x 10^5 exponential phase cells of a yeast strain. Each bottle also received an appropriate volume of sterile distilled water resulting in a soil moisture content equal to 100% field capacity (v/w), 25% (v/w), 10% (v/w) or 5% (v/w). To enumerate yeasts, soil dilutions plates with MEA containing (per liter of distilled water) 15 g of malt extract and 20 g of agar were periodically prepared. The plates were incubated at 30^o C and the colonies were counted after 2-3 days of incubation. Sampling was repeated on day 3, 5, 7, 10 and 20. All experiments were conducted in triplicate.

4. Results

4.1. Growth responses of basidiomycetous yeasts to osmotic stress

The growth of 41 basidiomycetous yeasts when the a_w was adjusted with either NaCl or sorbitol from 0.998 to 0.875 a_w are shown in Figures 7.1 (Appendix). As the a_w was reduced, the growth of the yeast declined. The experiments were conducted for up to 90 days as this was deemed long enough to ascertain whether the yeast could commence to grow. The yeasts varied considerably in their growth patterns. In the presence of NaCl some yeasts were more sensitive than other yeasts (Figures 7.1). For example *Cryptococcus bhutanesis* CBS 6294T had a minimum growth a_w at 0.925 adjusted with either NaCl or sorbitol, while *Cryptococcus albidus* CBS 5737 had a minimum growth a_w at 0.925 when adjusted with sorbitol and tolerated a minimum a_w with NaCl at 0.90.

Growth in strains of the same species, namely *C. laurentii* (3 strains; Figures 7.1.2, 7.1.4, 7.1.9, 7.1.10), *Cryptococcus neoformans* (7 strains; Figures 7.1.2-3, 7.1.9-10) and *Filobasidiella neoformans* (3 strains; Figures 7.1.4-5, 7.1.12) showed different responses to a_w when adjusted with either NaCl or sorbitol. For example, *C. laurentii* US 1A and US 1F strains had a minimum growth a_w of 0.95 when adjusted with NaCl or sorbitol, whereas *C. laurentii* CBS 0139 had lower minimum a_w (0.925). The minimum growth a_w (NaCl) of *C. neoformans* US I1 and *C. neoformans* US S5 was 0.925 whereas the minimum growth a_w (NaCl) of the other 5 strains was 0.95 a_w. When the a_w adjusted with sorbitol, the minimum growth a_w of all the *C. neoformans* strains was 0.95 a_w with the exception of *C. neoformans* US C2 which was 0.975 a_w. The minimum growth for all the *F. neoformans* strains was 0.95 a_w (sorbitol) but when strains were exposed to a_w adjusted with NaCl the minimum growth a_w of strains CBS 6885 and CBS 0884 was 0.925 a_w whereas strain CBS 0132 was 0.95 a_w. Other differences in minimum growth a_w were noted between strains of *Filobasidium floriforme, Filobasidium capsuligenum, Rhodotorula minuta* and *Sterigmatomyces halophilus.*

No basidiomycetous yeast was found to have a minimum a_w less than 0.9 (Table 4.1). This is in contrast to the ascomycetous yeasts where a minimum growth a_w of 0.65 was determined for *Z. rouxii* and the minimum growth a_w of many yeasts was found to be less than 0.9 (Van Eck et al., 1993).

4.2. Identification of intracellular solutes by ^{13}C NMR spectroscopy

When the basidiomycetous yeast were grown in the presence of NaCl (0.95 a_w), the peak profiles of the ^{13}C NMR spectrographs changed compared to growth at 0.998 a_w in Figures 7.2 (Appendix). The profiles of yeasts grown under NaCl stress were generally found to have fewer notable peaks when compared with yeasts grown without stress. Therefore, the ^{13}C NMR spectrograph can be used as an indication of change in concentrations or absence/presence of most metabolites. However, ^{13}C NMR spectroscopy should be applied as a qualitative method in the analysis of chemical compounds in yeast and the insensitivity of the method precludes detection in the changes in low metabolite concentrations (Friebolin, 1988). Identification of the main peaks as detected by ^{13}C NMR spectroscopy (Figures 4.2) revealed that these peaks are the osmolytes accumulated by yeast in response to osmotic stress (0.95 a_w NaCl) imposed on the yeast during growth. The main osmolytes identified by ^{13}C NMR spectroscopy were glycerol, arabitol, mannitol and trehalose and were confirmed by HPLC. However, as a result of variation in the values obtained for these osmolytes, accurate determination of the intracellular concentrations of these compounds using HPLC was unsuccessful. In addition, osmolyte concentrations below the detection level of ^{13}C NMR spectroscopy could not be measured.

While variation in the accumulation of arabitol, mannitol and trehalose between the yeast strains was observed, all strains were found to accumulate glycerol (Figures 4.2.1-10; Table 4.2). Arabitol was accumulated by 21 strains of yeasts (Table 4.2). However, 5 strains that, includes *C laurentii* 132T, *C. neoformans* US C2 and *F. unigetulattum* CBS 2770 yeasts accumulated mannitol. Furthermore, 4 strains, namely *C. curvatus* 2176, *F. capsuligenum* CBS 4381, *F. capsuligenum* CBS 6122.2 and *F. floriforme* CBS 6240, accumulated trehalose, although the role of trehalose in response to osmotic stress is not always clear (see discussion). In some yeasts, only glycerol was found to be accumulated, namely *Cryptococcus macerans* CBS 2206T, *Cryptococcus gastricus* CBS 1927, *C. neoformans* US I5, *C. neoformans* I6, *F. neoformans* CBS 0884, *F. neoformans* CBS 6885, *Rhodotorula araucariae* CBS 6031T, *Rhodotorula graminis* CBS 2826, *Rhodotorula lactosa* CBS 5826T, *Rhodotorula mucilaginosa* CBS 5951 and *Rhodotorula sp* CBS 5143. More sensitive analytical techniques would be required to ascertain whether these yeasts accumulated other osmolytes.

It was apparent that all yeasts except *C. laurentii* strain US 1F (Figure 4.2.3A) accumulated just one osmolyte in addition to glycerol. Some differences in osmolyte accumulation were noted between

strains. *C. laurentii* strain US 1F (Fig. 4.2.3A) was found to accumulate trehalose in addition to glycerol and mannitol whereas another two strains of *C. laurentii* US 1F and CBS 0139 (Fig. 4.2.3A) accumulated only glycerol and mannitol or arabitol respectively. All seven *C. neoformans* strains accumulated glycerol but varied in the accumulation of either arabitol or mannitol (Table 4.2). However some species were consistent in the accumulation of their osmolytes. For example, both strains of *F. capsuligenum*, *R. minuta* and *S. halophilus*, respectively, accumulated glycerol/trehalose, glycerol/arabitol and glycerol/arabitol (Table 4.2). Nevertherless, *F. neoformans* strains accumulated different osmolytes, whereby two strains (CBS 0884, and CBS 6885) accumulated glycerol only and one strain (CBS 0132) accumulated arabitol in addition to glycerol (Table 4.2).

Table 4.1. Minimum water activity (a_w) for growth of basidiomycetous yeasts

Species	Accession source and Number	NaCl	Sorbitol
Bulleromyces albus	CBS 6097 (CSIR Y-498)	0.9	0.925
Bullera dendrophila	CBS 6074T (CSIR Y-499)	0.95	0.95
Cryptococcus albidus	CBS 5737 (CSIR Y-73)	0.9	0.925
Cryptococcus amylolentus	CBS 6039T (CSIR Y-501)	0.9	0.95
Cryptococcus bhutanensis	CBS 6294T	0.925	0.925
Cryptococcus curvatus	CBS 2176	0.9	0.925
Cryptococcus gastricus	CBS 1927	0.95	0.95
Cryptococcus hungaricus	CBS 5124 (CSIR Y-548)	0.925	0.975
Cryptococcus laurentii	US 1A	0.95	0.95
Cryptococcus laurentii	US 1F	0.95	0.95
Cryptococcus laurentii	CBS 0139	0.925	0.925
Cryptococcus macerans	CBS 2206T	0.95	0.925
Cryptococcus neoformans	US 132T	0.95	0.95
Cryptococcus neoformans	US C2	0.95	0.975
Cryptococcus neoformans	US I1	0.925	0.95
Cryptococcus neoformans	US I5	0.95	0.95
Cryptococcus neoformans	US I6	0.95	0.95
Cryptococcus neoformans	US S5	0.925	0.95
Cryptococcus neoformans	US I4	0.95	0.95
Cryptococcus podzolicus	US 5A	0.9	0.925
Cryptococcus terreus	CBS 1895T	0.925	0.95
Filobasidium capsuligenum	CBS 4381	0.925	0.925
Filobasidium capsuligenum	CBS 6122.2	0.95	0.975
Filobasidium floriforme	CBS 6240	0.95	0.90
Filobasidiella neoformans	CBS 0132	0.95	0.95
Filobasidiella neoformans	CBS 0884	0.925	0.95
Filobasidiella neoformans	CBS 6885	0.925	0.95
Filobasidium unigetulattum	CBS 2770	0.9	0.925
Rhodotorula araucariae	CBS 6031T	0.95	0.95
Rhodosporidium glutinis	CBS 0020	0.925	0.925
Rhodotorula graminis	CBS 2826	0.9	0.925
Rhodotorula lactosa	CBS 5826T	0.9	0.925
Rhodotorula minuta	CBS 2172	0.9	0.95
Rhodotorula minuta	CBS 2177	0.95	0.95
Rhodotorula mucilaginosa	CBS 5951	0.95	0.975
Rhodotorula toruloides	CBS 0349	0.925	0.95
Rhodotorula sp	CBS 5143 (UF-448)	0.95	0.95
Sterigmatomyces halophilus	CBS 4609Y (N-4619T)	0.9	0.925
Sterigmatomyces halophilus	CBS 5628Y (N-6837))	0.925	0.95
Sporidiobolus salmonicolor	CBS 5937Y (CSIR Y-144)	0.9	0.925
Trichosporon cuteneum	CBS 2644NT (CSIR Y-351)	0.95	0.95

Table 4.2. Osmolytes identified by ^{13}C NMR spectroscopy when yeasts were exposed to osmotic stress (0.95 a_w NaCl)

		Osmolytes			
Species	Accession source and number	Glycerol	Arabitol	Mannitol	Trehalose
Bulleromyces albus	CBS 6097 (CSIR Y-498)	+	+	-	-
Bullera dendrophila	CBS 6074T (CSIR Y-499)	+	+	-	-
Cryptococcus albidus	CBS 5737 (CSIR Y-73)	+	+	-	-
Cryptococcus amylolentus	CBS 6039T (CSIR Y-501)	+	+	-	-
Cryptococcus bhutanensis	CBS 6294T	+	+	-	-
Cryptococcus curvatus	CBS 2176	+	-	-	+
Cryptococcus gastricus	CBS 1927	+	-	-	-
Cryptococcus hungaricus	CBS 5124 (CSIR Y-548)	+	+	-	-
Cryptococcus laurentii	US 1A	+	-	+	-
Cryptococcus laurentii	US 1F	+	-	+	+
Cryptococcus laurentii	CBS 0139	+	+	-	-
Cryptococcus macerans	CBS 2206T	+	-	-	-
Cryptococcus neoformans	US 132T	+	-	+	-
Cryptococcus neoformans	US C2	+	-	+	-
Cryptococcus neoformans	US I1	+	+	-	-
Cryptococcus neoformans	US I5	+	-	-	-
Cryptococcus neoformans	US I6	+	-	-	-
Cryptococcus neoformans	US S5	+	+	-	-
Cryptococcus neoformans	US I4	+	-	+	-
Cryptococcus podzolicus	US 5A	+	+	-	-
Cryptococcus terreus	CBS 1895T	+	+	-	-
Filobasidium capsuligenum	CBS 4381	+	-	-	+
Filobasidium capsuligenum	CBS 6122.2	+	-	-	+
Filobasidium floriforme	CBS 6240	+	-	-	+
Filobasidiella neoformans	CBS 0132	+	+	-	-
Filobasidiella neoformans	CBS 0884	+	-	-	-
Filobasidiella neoformans	CBS 6885	+	-	-	-
Filobasidium unigetulattum	CBS 2770	+	-	+	-
Rhodotorula araucariae	CBS 6031T	+	-	-	-
Rhodosporidium glutinis	CBS 0020	+	+	-	-
Rhodotorula graminis	CBS 2826	+	-	-	-
Rhodotorula lactosa	CBS 5826T	+	-	-	-
Rhodotorula minuta	CBS 2172	+	+	-	-
Rhodotorula minuta	CBS 2177	+	+	-	-
Rhodotorula mucilaginosa	CBS 5951	+	-	-	-
Rhodotorula toruloides	CBS 0349	+	+	-	-
Rhodotorula sp	CBS 5143 (UF-448)	+	-	-	-
Sterigmatomyces halophilus	CBS 4609Y (N-4619T)	+	+	-	-
Sterigmatomyces halophilus	CBS 5628Y (N-6837))	+	+	-	-
Sporidiobolus salmonicolor	CBS 5937Y (CSIR Y-144)	+	+	-	-
Trichosporon cuteneum	CBS 2644NT (CSIR Y-351)	+	+	-	-

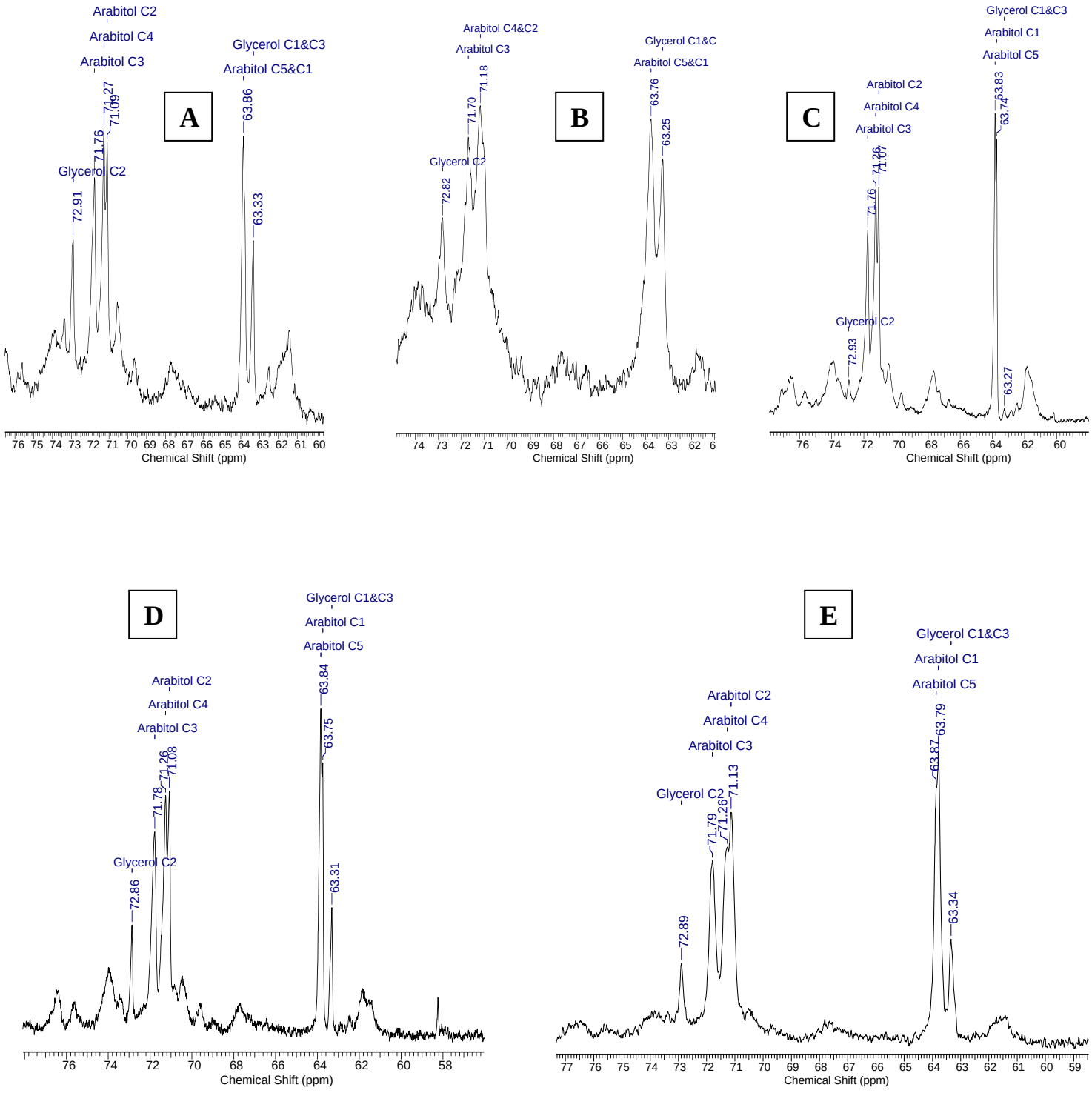

Figure 4.2.1: The compounds identified by ^{13}C NMR spectroscopy from (A) *B. albus* CBS 6097, (B) *B. dendrophila* CBS 6074T, (C) *C. albidus* CBS 5737 (D) *C. amylolentus* CBS 6039T and (E) *C. hungaricus* CBS 5124when exposed to osmotic stress (0.95 a_w NaCl).

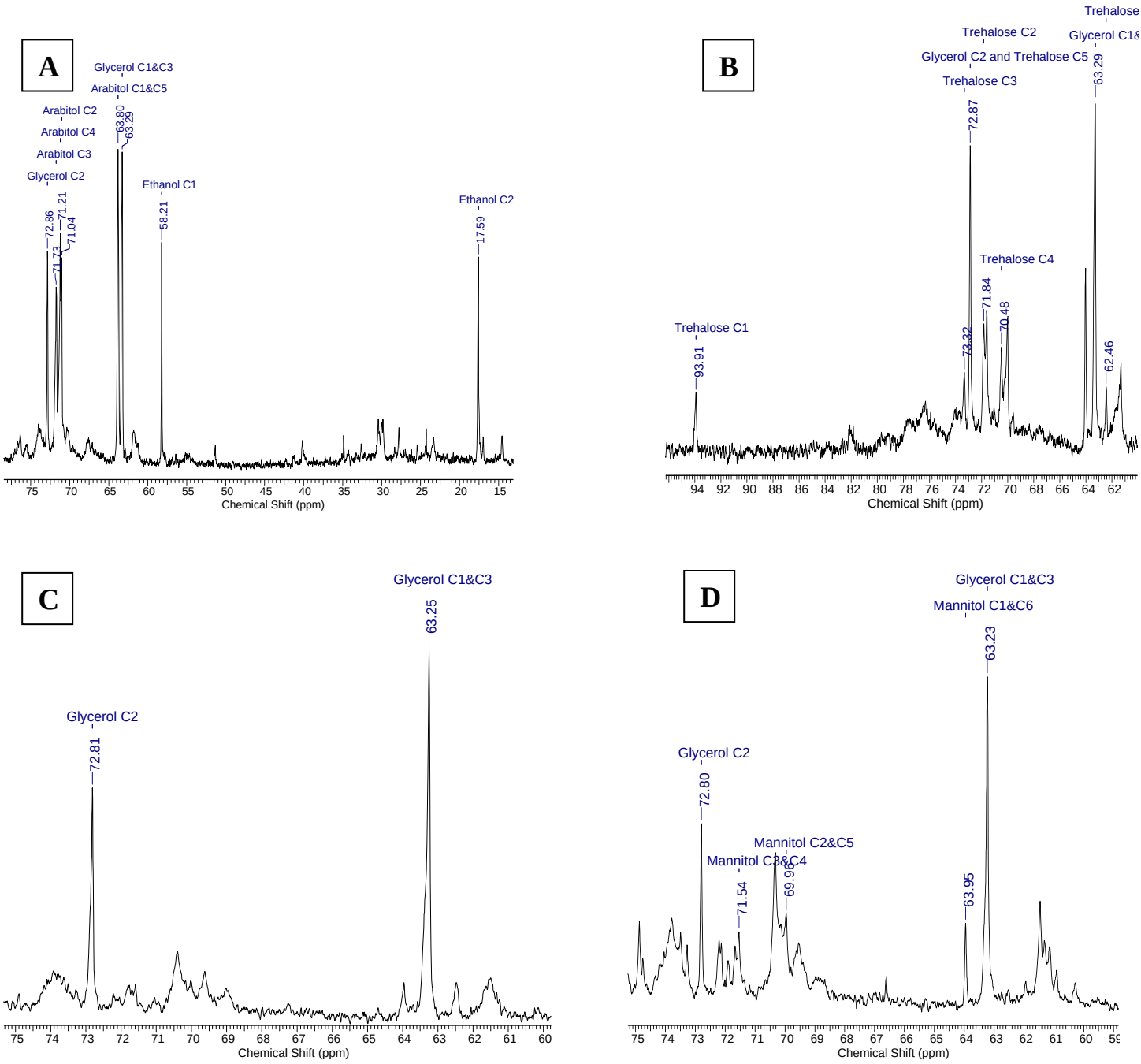

Figure 4.2.2: The compounds identified by ^{13}C NMR spectroscopy from (A) *C. bhutanensis* CBS 6294T, (B) *C. curvatus*, CBS 2176 (C) *C. gastricus* CBS 1927 and (D) *C. laurentii* US 1A when exposed to osmotic stress (0.95 a_w NaCl).

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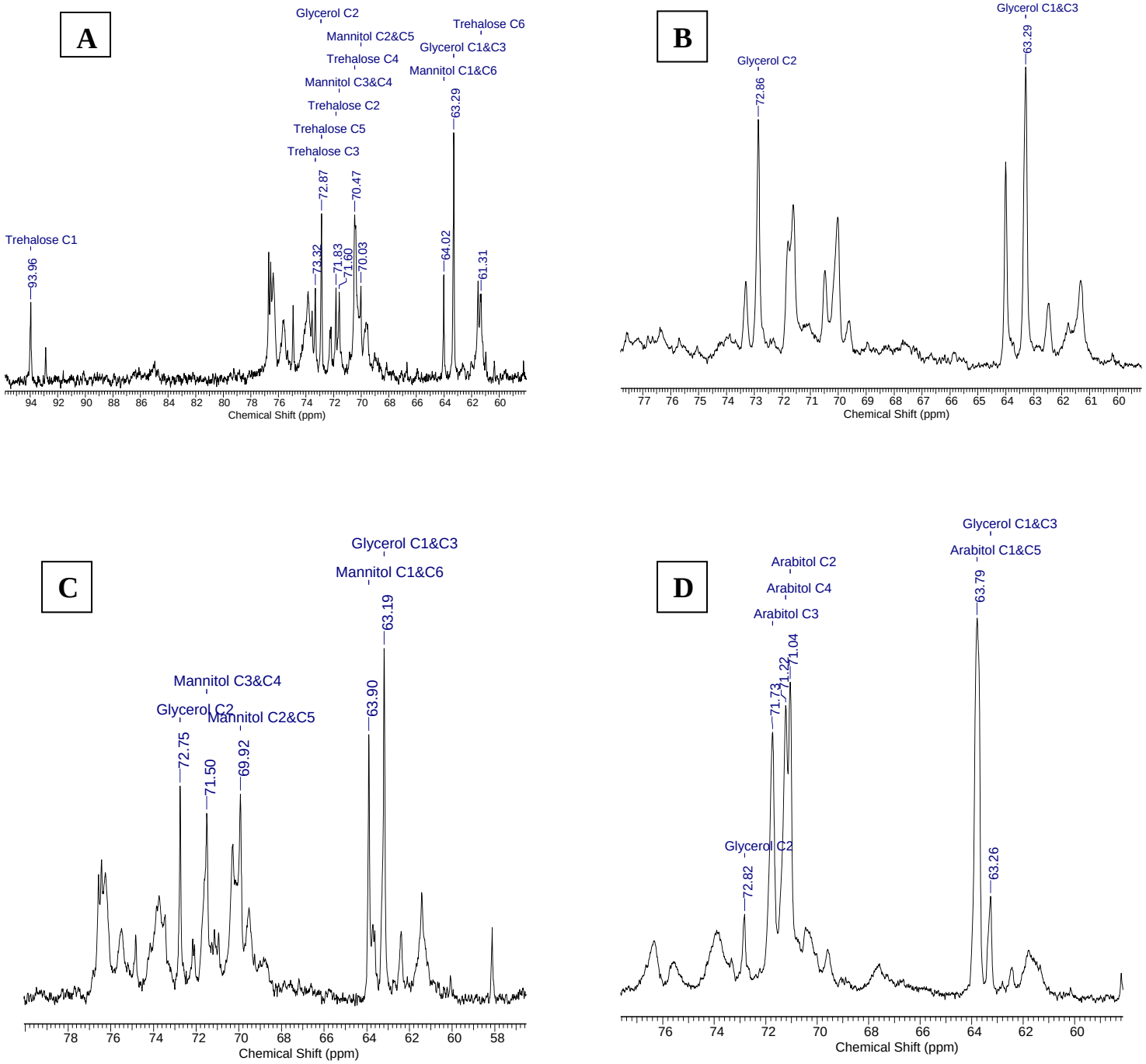

Figure 4.2.3: The compounds identified by ^{13}C NMR spectroscopy from (A) *C. laurentii* US 1F, (B) *C. macerans* CBS 2206T, (C) *C. neoformans* US C2 and (D) *C. neoformans* US I1 when exposed to osmotic stress (0.95 a_w NaCl).

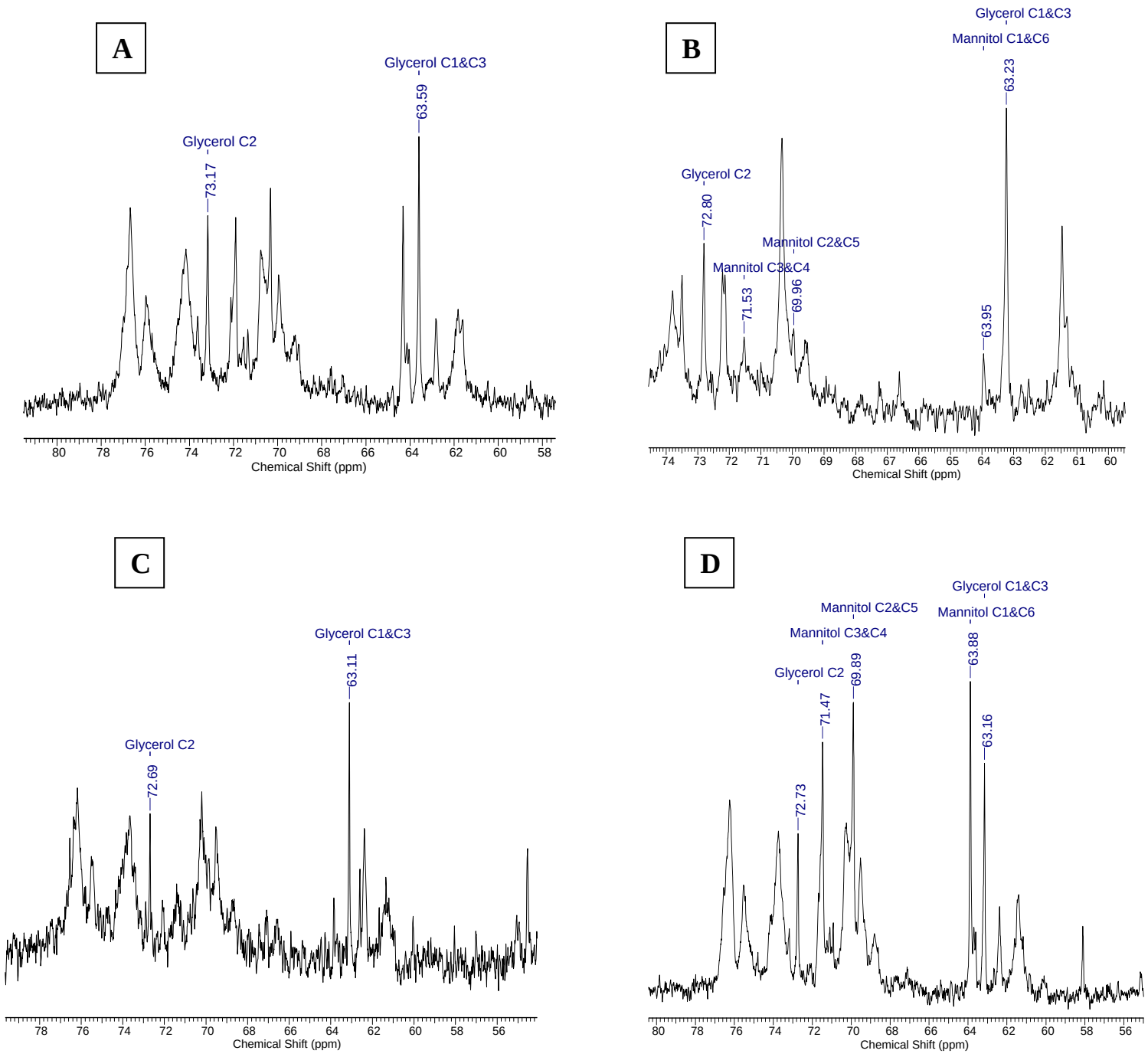

Figure 4.2.4: The compounds identified by ^{13}C NMR spectroscopy from (A) *C. neoformans* US I5, (B) *C. neoformans* US 132T (C) and (C) *C. neoformans* US I6, (D) *C. neoformans* US I4 when exposed to osmotic stress (0.95 a_w NaCl).

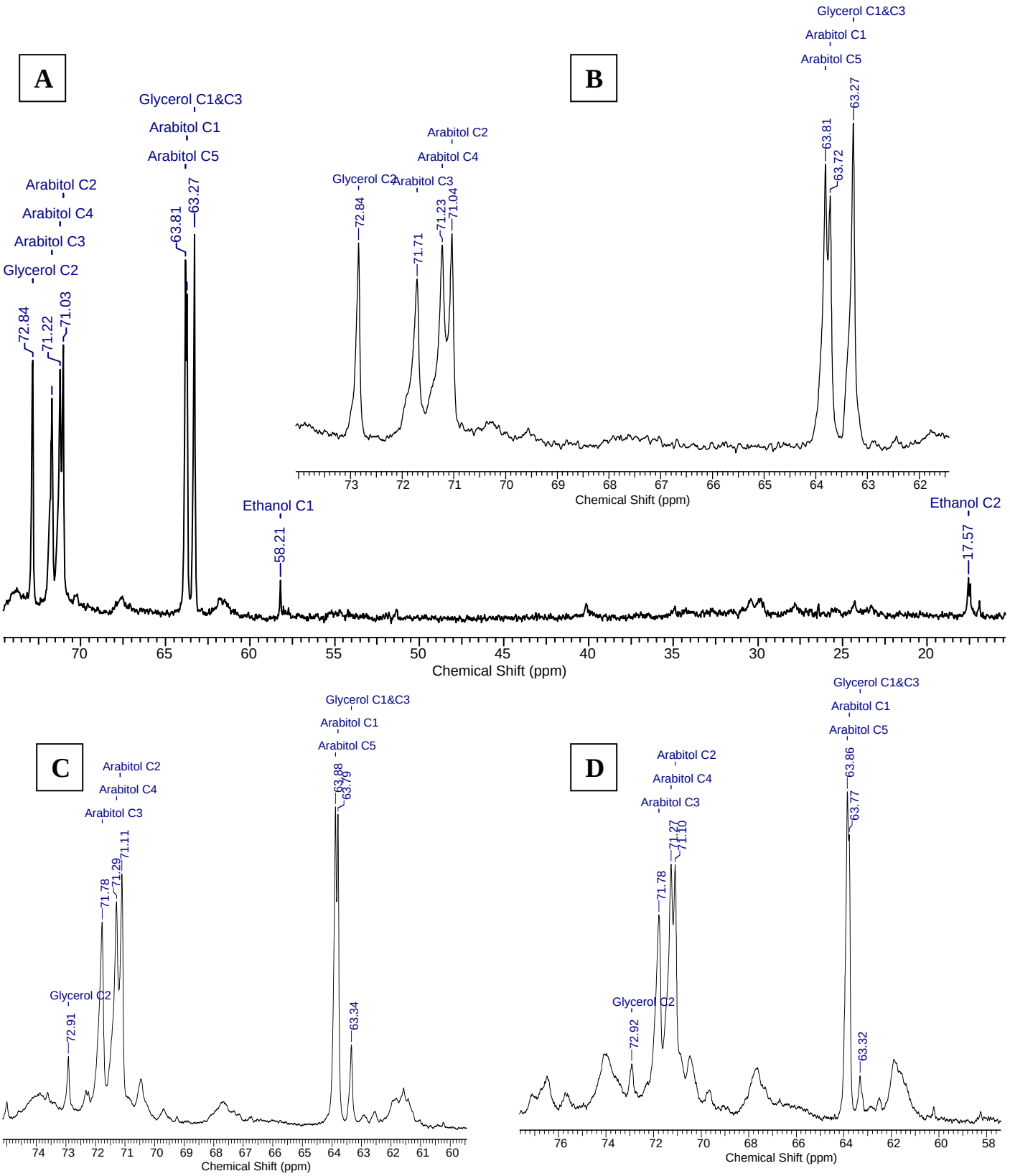

Figure 4.2.5: The compounds identified by ^{13}C NMR spectroscopy from (A) *C. neoformans* US S5, (B) *C. podzolicus* US 5A, (C) *C. terreus* CBS 1895T, and (D) *C. laurentii* CBS 0139 when exposed to osmotic stress(0.95a_w NaCl).

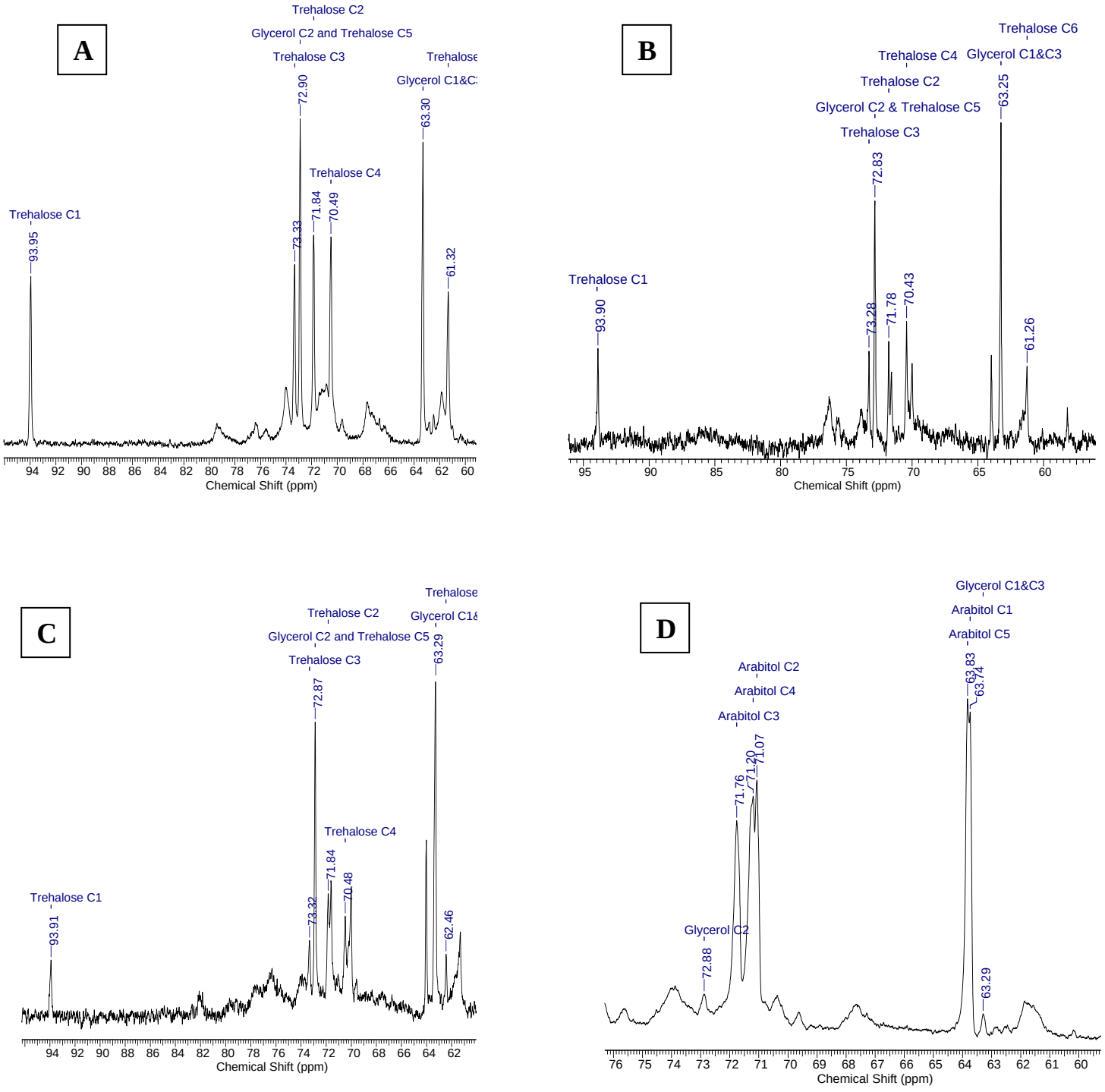

Figure 4.2.6: The compounds identified by ^{13}C NMR spectroscopy from (A) *F. capsuligenum* CBS 4381, (B) *F. capsuligenum* CBS 6122.2, (C) *F. floriforme* CBS 6240, (D) *F. neoformans* CBS 0132 when exposed to osmotic stress (0.95 a_w NaCl).

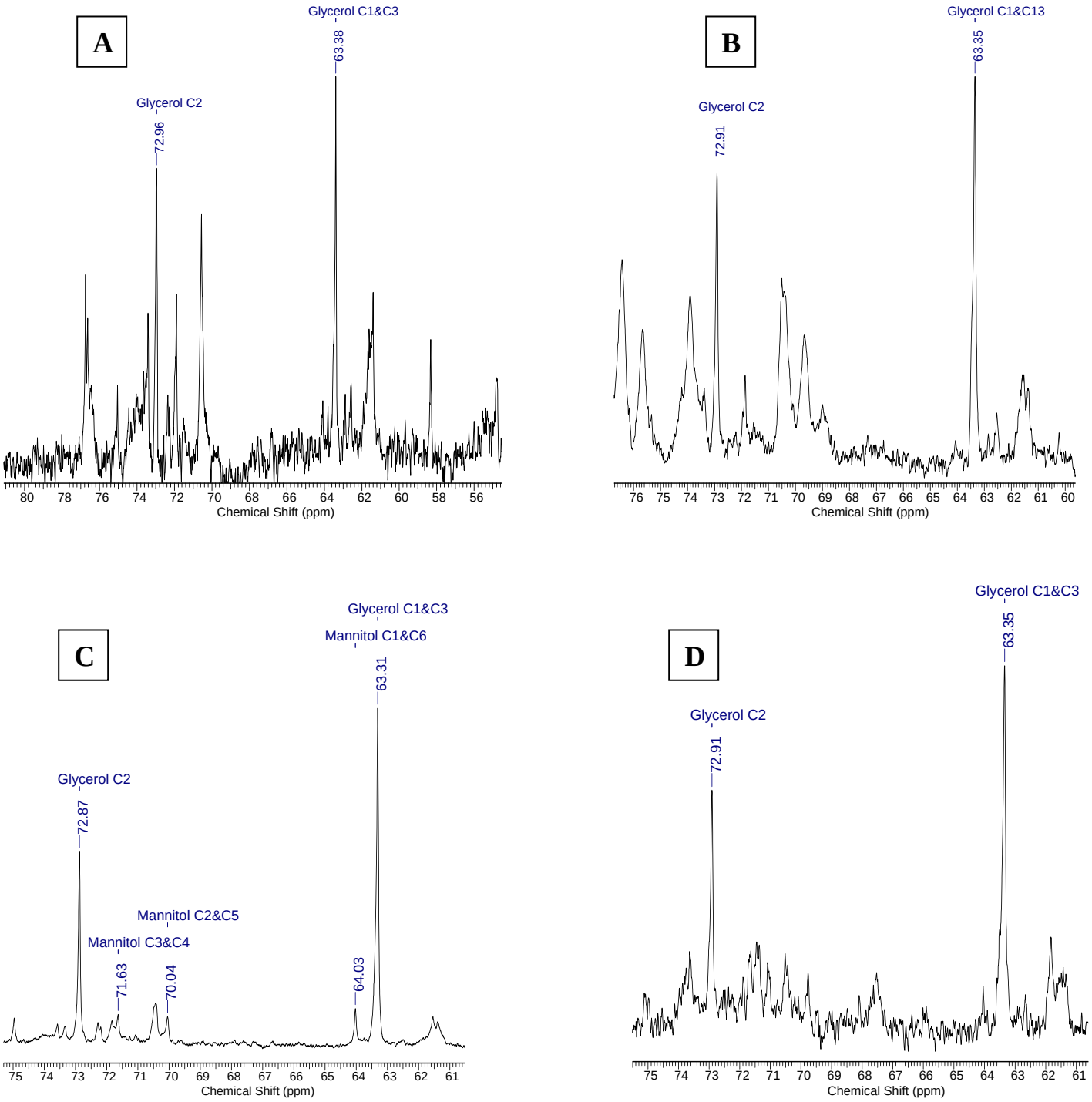

Figure 4.2.7: The compounds identified by ^{13}C NMR spectroscopy from (A) *F. neoformans* CBS 0884, (B) *F. neoformans* CBS 6885, (C) *F unigetulattum* CBS 2770, (D) *R. araucariae* 6031T when exposed to osmotic stress (0.95 a_w NaCl).

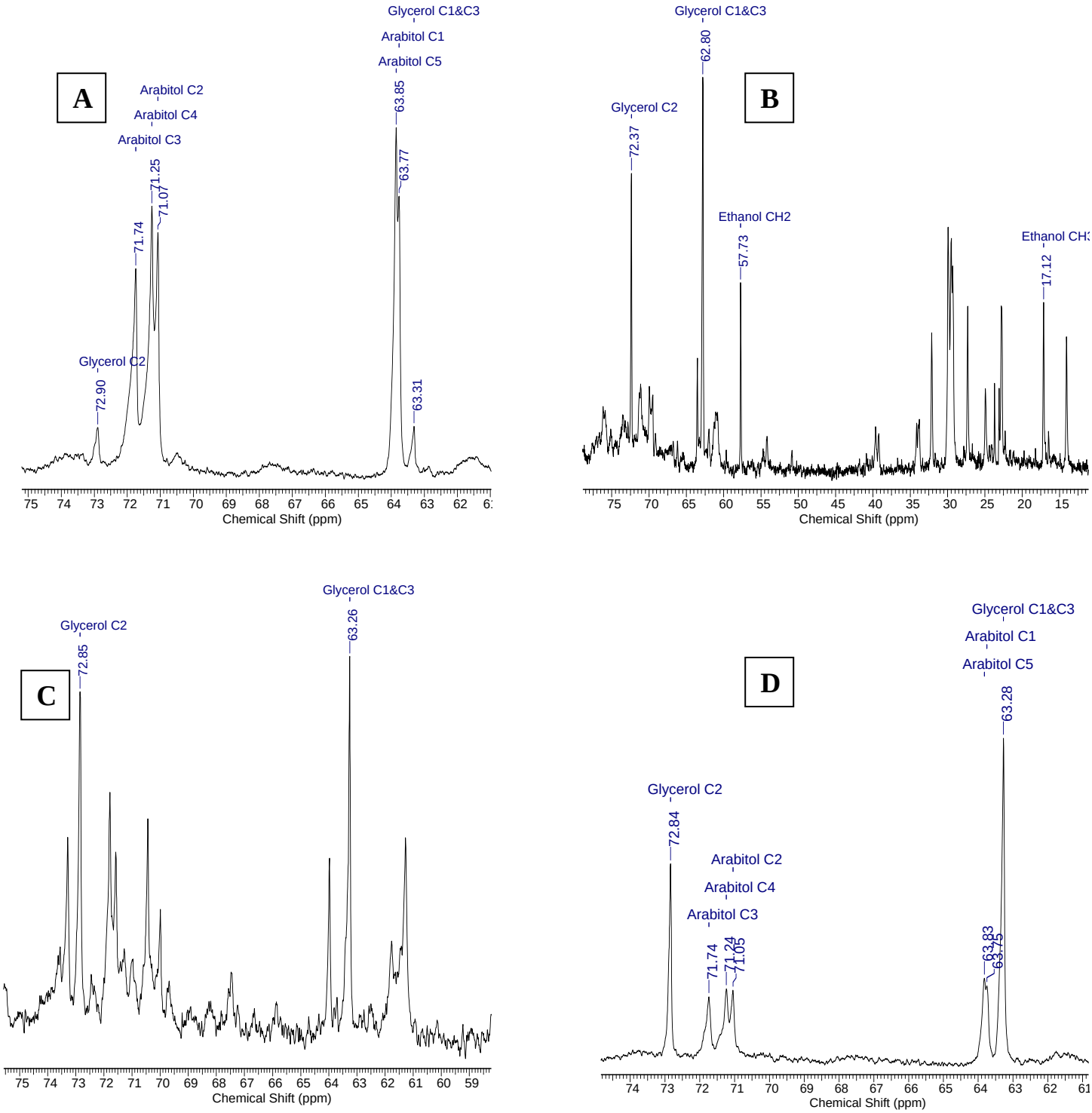

Figure 4.2.8: The compounds identified by ^{13}C NMR spectroscopy from (A) *R. glutinis* CBS 0020, (B) *R. graminis* CBS 2826T, (C) *R. lactosa* CBS 5826 (D) *R. toruloides* CBS 0349 when exposed to osmotic stress (0.95 a_w NaCl).

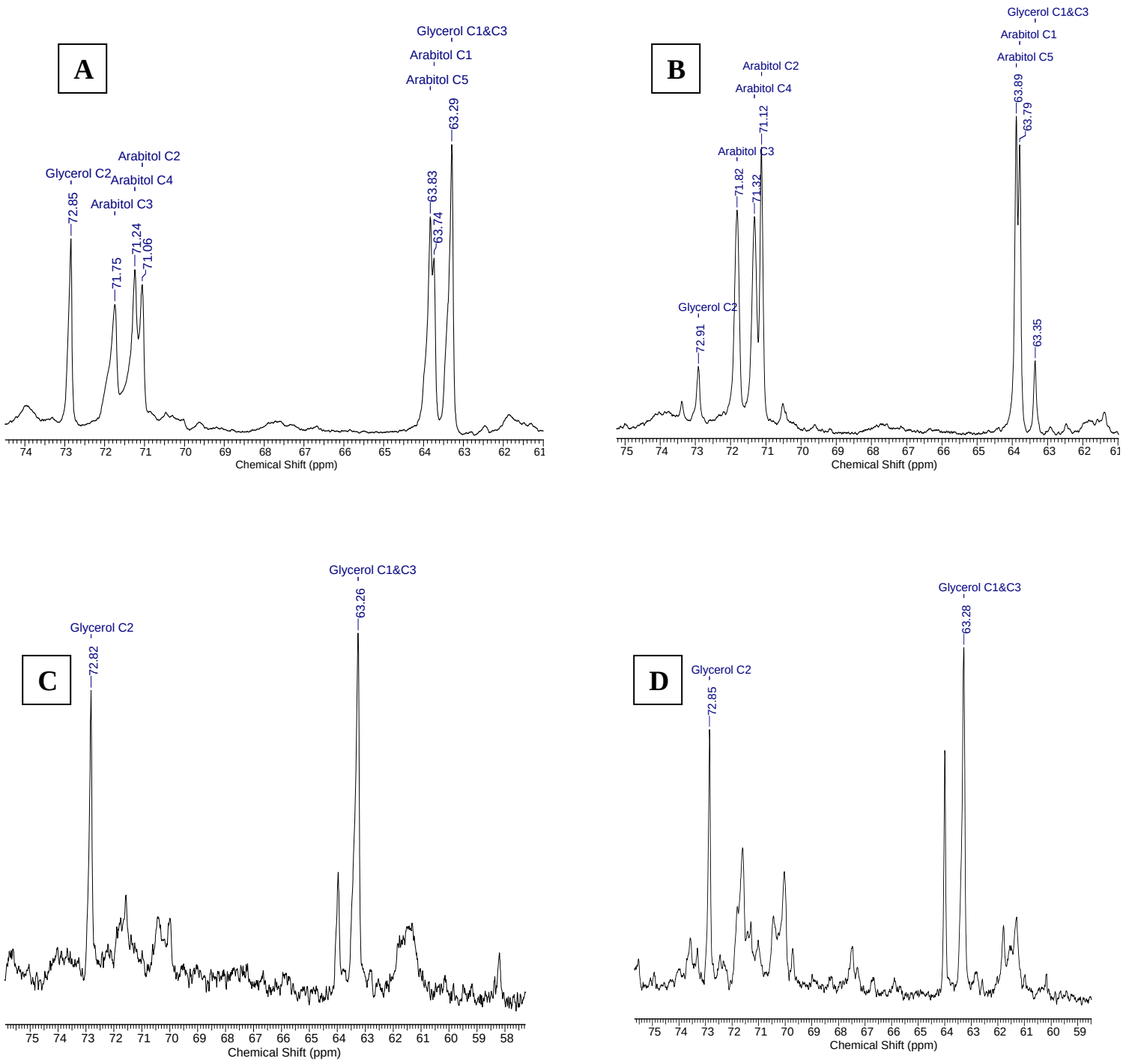

Figure 4.2.9: The compounds identified by ^{13}C NMR spectroscopy from (A) *R. minuta* var *minuta* CBS 2172, (B) *R. minuta* var *minuta* CBS 2177 (C) *R. mucilaginosa* CBS 5951 (D) *Rhodotorula* sp CBS 5143 when exposed to osmotic stress (0.95 a_w NaCl).

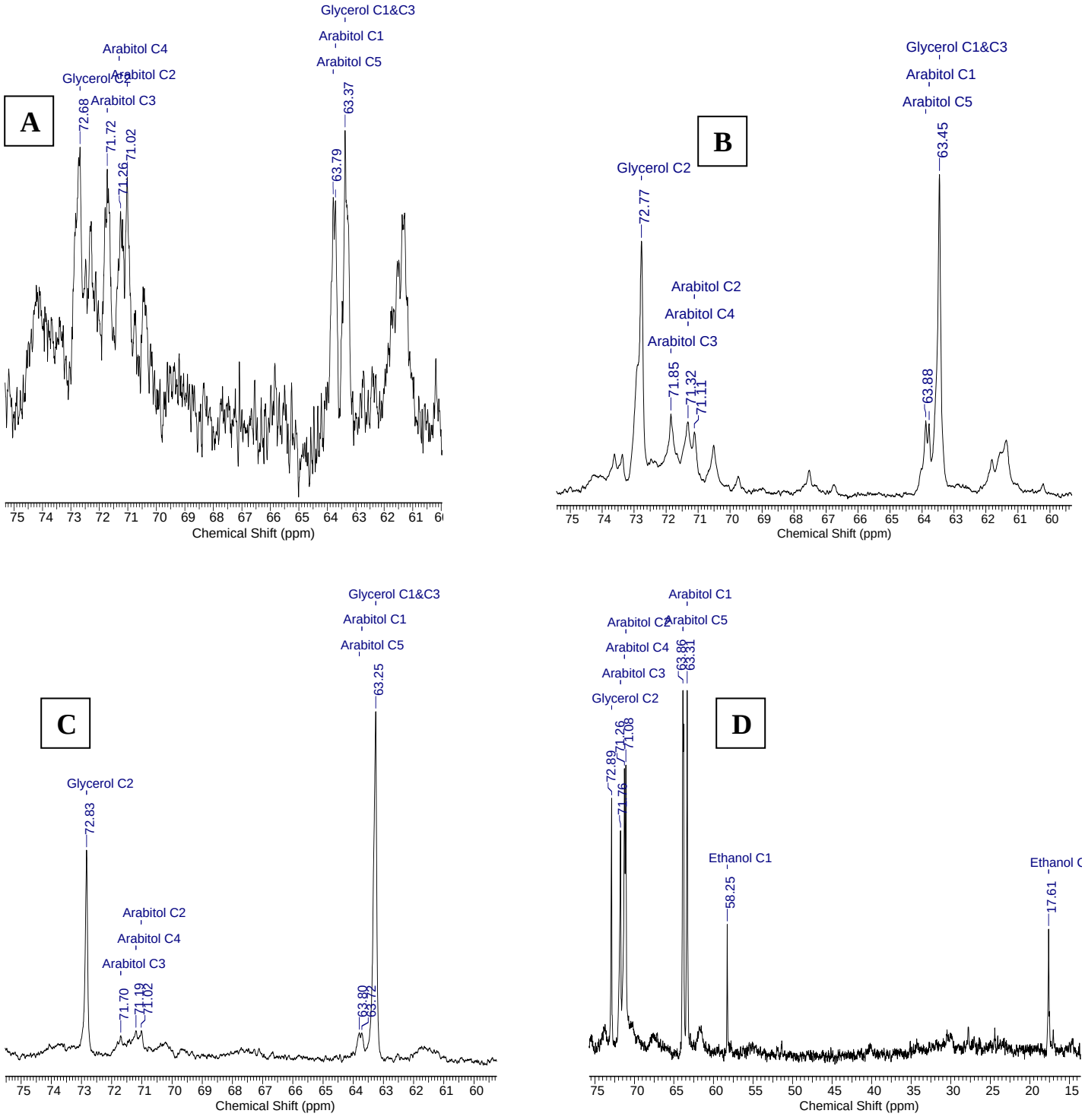

Figure 4.2.10: The compounds identified by ^{13}C NMR spectroscopy from (A) *S. halophilus* CBS 4609Y, (B) *S. halophilus* CBS 5628Y, (C) *S. salmonicolor* CBS 5937, (D) *T. cuteneum* CBS 2466NT when exposed to osmotic stress (0.95 a_w NaCl).

4.3. Effect of stage of growth on intracellular solute accumulation as identified by ^{13}C NMR spectroscopy and HPLC

Figs 4.3.1-3 show the ^{13}C NMR spectroscopy of three yeasts (*C. hungaricus* CBS 5124, *C. neoformans* US I1 and *C. macerans* CBS 2206T) when the samples were taken at various stages of growth at $0.95a_w$ (NaCl). This was done to ascertain whether the type of osmolyte accumulated may vary with growth stage. No changes in the type of osmolyte present were observed. The ^{13}C NMR spectrographs of *C. hungaricus* CBS 5124 (Figure 4.3.1) revealed that the proportions of glycerol to arabitol changed from samples taken at 12 h to that at 30 h. Glycerol concentrations declined while arabitol increased. Similar changes were also observed with the ^{13}C NMR spectrographs of *C. neoformans* US I1 from 8 h to 30 h. With *C. macerans* CBS 2206T (Fig. 4.3.2) glycerol remained as the only detectable osmolyte in samples taken from 8 to 26 h of growth at $0.95a_w$ (NaCl) and no other osmolytes were observed at the level of sensitivity of the ^{13}C NMR spectrometer. These results together with those reported in section 4.2 confirm that glycerol is the major osmolyte accumulated by the basidiomycetous yeast strains used in this study.

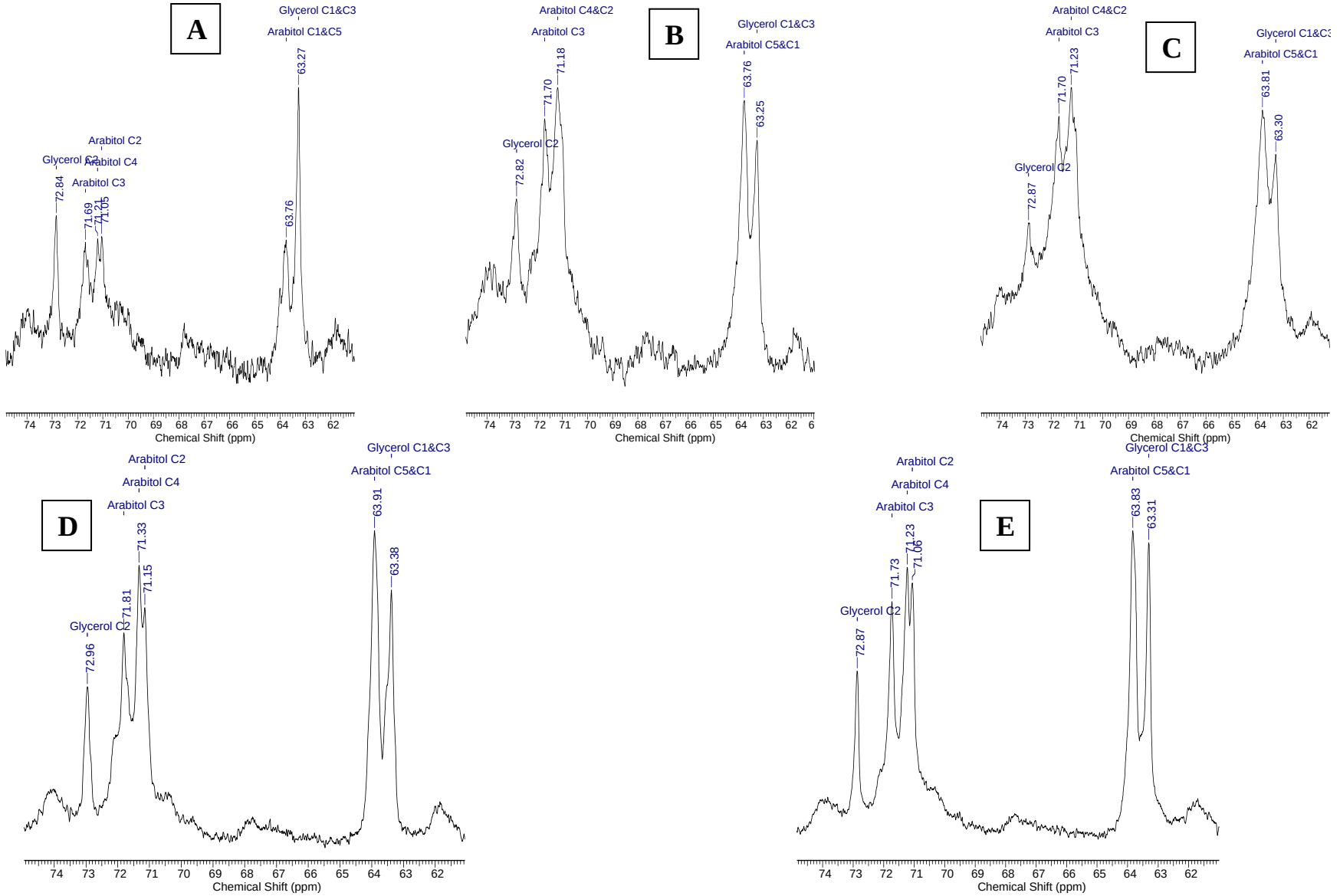

Figure 4.3.1: The compounds identified by ^{13}C NMR spectroscopy from *C. hungaricus* CBS 5124 at (A)12h, (B) 18h (C) 22h (D) 26h and (E) 30h when exposed to osmotic stress (0.95 a_w NaCl).

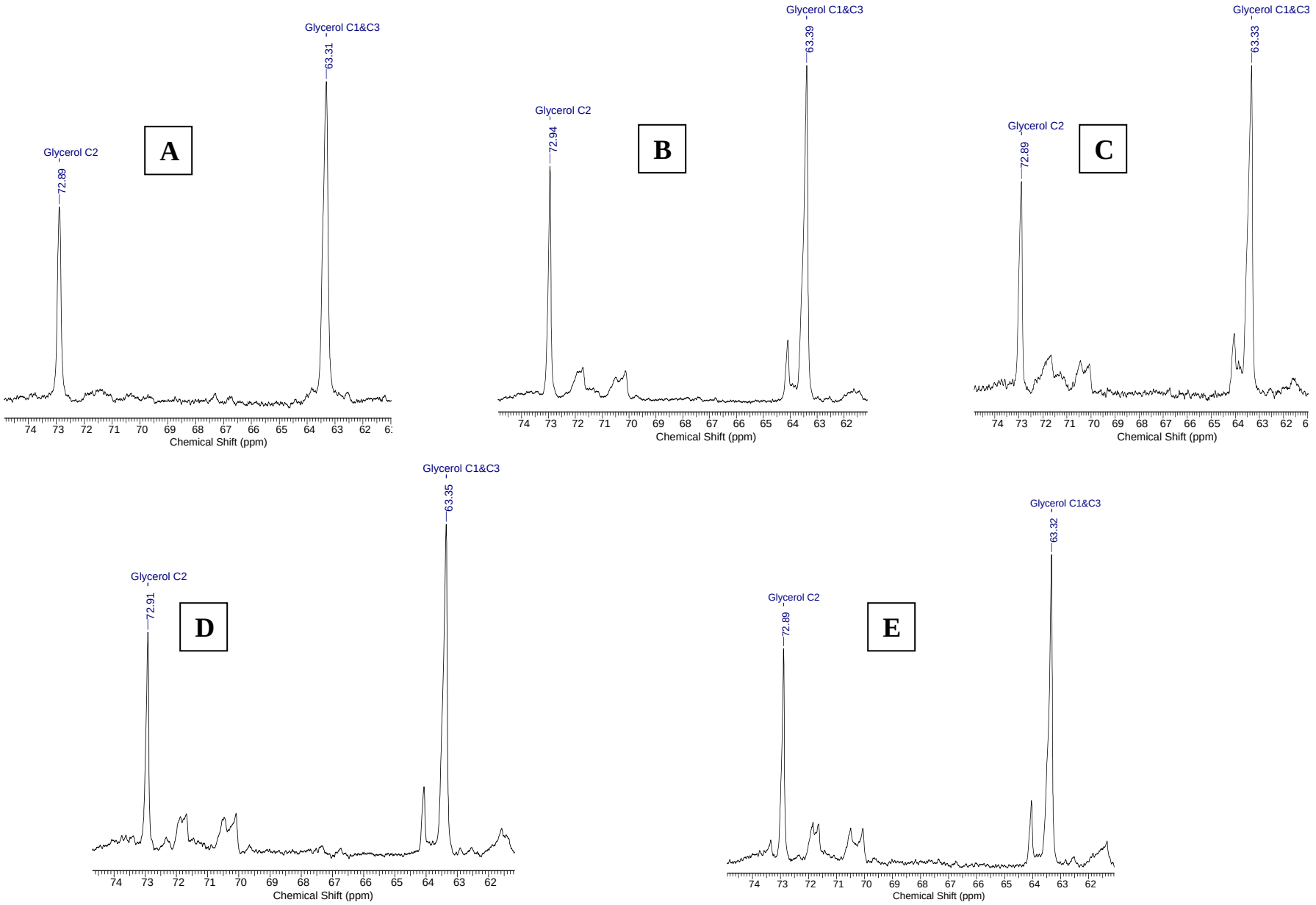

Figure 4.3.2: The compounds identified by ^{13}C NMR spectroscopy from *C. macerans* CBS 2206T at (A) 8h, (B) 14h (C) 18h (D) 22h and (E) 26h when exposed to osmotic stress (0.95 a_w NaCl).

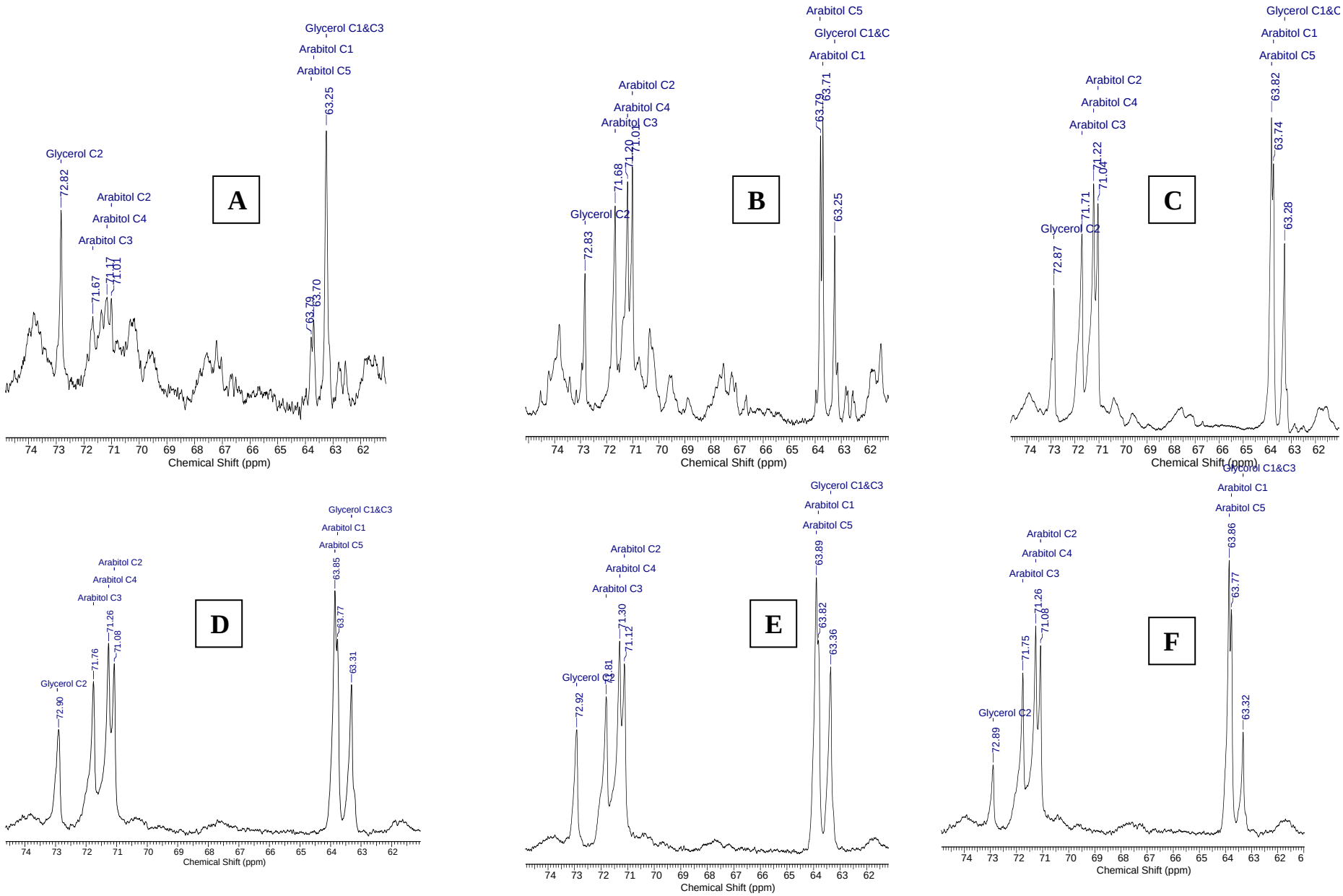

Figure 4.3.3: The compounds identified by ^{13}C NMR spectroscopy from *C. neoformans* US I1 at (A) 8h, (B) 14h (C) 18h (D) 22h (E) 26h and (F) 30h when exposed to osmotic stress (0.95 a_w NaCl)

4.4. Response of selected yeasts to hypo-osmotic stress

Table 4.3 shows that most yeasts survived hypo-osmotic stress without a loss of viability. Only *B. albus* CBS 6097, *C. laurentii* US 1A, *C. macerans* CBS 2206T, *C. neoformans* US I5, *F. unigetulattum* CBS 2770 and *S. salmonicolor* CBS 5951 showed a decline in viability and in most cases the decline was not substantial. This observation suggests that the basidiomycetous yeasts must have efficient mechanisms to cope with or dispose of the accumulated osmolytes. Previous work with *S. cerevisiae* revealed that this yeast has a channel protein to export glycerol (Luyten et al., 1995) and subsequent work by Tang et al. (2006) confirmed that *Z. rouxii* also possessed such an osmolyte export channel that was able to export glycerol and arabitol when subject to hypo-osmotic stress. Without this channel protein, the yeasts loose viability rapidly.

No clear picture of the release of osmolytes was obtained when five basidiomycetous yeasts were subject to hypo-osmotic stress (Figure 4.4.1). *C. laurentii* strain US 1F (Fig. 4.4.1A) was found to rapidly release glycerol, mannitol and trehalose but nearly 60, 70 and 80% of the respective intracellular glycerol, mannitol and trehalose concentration remained in the cell. Upon hypo-osmotic stress *C. neoformans* strain US I1 (Fig. 4.4.1B) rapidly released both glycerol and arabitol to 45 % of the original intracellular concentration but surprisingly the intracellular concentrations of these osmolytes subsequently increased. With *C. macerans* strain CBS 2206T (Fig. 4.4.1E), both intracellular glycerol and trehalose concentrations decreased with hypo-osmotic stress within 5 min but glycerol levels later increased back to the original level whereas the trehalose level continued to decline. No significant decrease of intracellular glycerol concentration was found when *R. mucilaginosa* CBS 5951 (Fig. 4.4.1D) and *C. macerans* CBS 2206T (Fig. 4.4.1E) were subject to hypo-osmotic stress. *R. mucilaginosa* survived the stress whereas the survival rate of *C. macerans* CBS 2206T was nearly 50 % less than the control condition (Table 4.3).

Table 4.3. Survival of basidiomycetous yeast cells after a hypo-osmotic shock from 0.972 a_w (5% NaCl) to 0.998 a_w

Organisms	Accession source and number	Colony forming units (Mean ± standard deviation of triplicate determination) Iso-osmotic condition	Hypo-osmotic condition	% change (+/-)
Bulleromyces albus	CBS 6097 (CSIR Y-498)	215 ± 11.3	151 ± 24	-29.7
Bullera dendrophila	CBS 6074T (CSIR Y-499)	57 ± 14	114 ± 9	50
Cryptococcus albidus	CBS 5737 (CSIR Y-73)	66 ± 11.1	181 ± 12	63.5
Cryptococcus amylolentus	CBS 6039T (CSIR Y-501)	39 ± 5	95 ± 6	58.9
Cryptococcus bhutanensis	CBS 6294T	66 ± 11	114 ± 7	40.7
Cryptococcus curvatus	CBS 2176	79 ± 20.9	118 ± 13	33
Cryptococcus gastricus	CBS 1927	44 ± 22.8	74 ± 13	40.5
Cryptococcus hungaricus	CBS 5124 (CSIR Y-548)	62 ± 13	136 ± 14	54.4
Cryptococcus laurentii	US 1A	172 ± 55.9	36 ± 6	-79.1
Cryptococcus laurentii	US 1F	126 ± 7	185 ± 17	31.9
Cryptococcus laurentii	CBS 0139	64 ± 17	111 ± 11	42.3
Cryptococcus macerans	CBS 2206T	124 ± 9	65 ± 11	-47.6
Cryptococcus neoformans	US 132T	161 ± 34.6	177 ± 11	9.0
Cryptococcus neoformans	US C2	55.3 ± 6.5	117 ± 10	52.9
Cryptococcus neoformans	US I1	104 ± 6.2	160 ± 16	35
Cryptococcus neoformans	US I5	218 ± 13	187 ± 11	-14.2
Cryptococcus neoformans	US I6	73 ± 13.8	107 ± 16	31.8
Cryptococcus neoformans	US S5	75.3± 6.65	105 ± 7	28.6
Cryptococcus neoformans	US I4	77 ± 12.5	118 ± 33	34.7
Cryptococcus podzolicus	US 5A	39 ± 8	76 ± 10	48.7
Cryptococcus terreus	CBS 1895T	47 ± 9	108 ± 9	56.5
Filobasidium capsuligenum	CBS 4381	36 ± 9	88 ± 10	59.1
Filobasidium capsuligenum	CBS 6122.2	84 ± 20	161 ± 15	47.8
Filobasidium floriforme	CBS 6240	76 ± 12	134 ± 14	43.28
Filobasidiella neoformans	CBS 0132	79 ± 9	131 ± 13	39.7
Filobasidiella neoformans	CBS 0884	97.3 ± 14.5	173 ± 17	43.9
Filobasidiella neoformans	CBS 6885	66 ± 11.6	114 ± 7	42.1
Filobasidium unigetulattum	CBS 2770	105 ± 8.5	56 ± 7	-46.7
Rhodotorula araucariae	CBS 6031T	65 ± 18.2	213 ± 12	69.5
Rhodosporidium glutinis	CBS 0020	55 ± 11	116 ± 81	52.6
Rhodotorula graminis	CBS 2826	127± 21.6	182 ± 17	30.2
Rhodotorula lactosa	CBS 5826T	47 ± 9	98 ± 21	52.0
Rhodotorula minuta	CBS 2172	74 ± 2.5	97 ± 10	23.7
Rhodotorula minuta	CBS 2177	36.7 ± 7.8	113 ± 15	68.1
Rhodotorula mucilaginosa	CBS 5951	162 ± 19.9	208 ± 29	19.3

(**Table 4.3.** Cont.)

Survival of basidiomycetous yeast cells after a hypo-osmotic shock from 0.972 a_w to 0.998 a_w

Organisms	Accession source and number	Colony forming units (Mean ± standard deviation of triplicate determination) Iso-osmotic condition	Hypo-osmotic condition	% change (+/-)
Rhodotorula toruloides	CBS 0349	108 ± 10.7	157 ± 11	31.2
Rhodotorula sp	CBS 5143 (UF-448)	76.33 ± 19.8	94.67 ± 19	19.2
Sterigmatomyces halophilus	CBS 4609Y (N-4619T)	113 ± 9	188 ± 15	39.9
Sterigmatomyces halophilus	CBS 5628Y (N-6837))	208 ± 10.6	238 ± 10	12.6
Sporidiobolus salmonicolor	CBS 5937Y (CSIR Y-144)	242 ± 5.51	184 ± 14	-23.9
Trichosporon cuteneum	CBS 2644NT (CSIR Y-351)	69 ± 7	105 ± 7	34.2

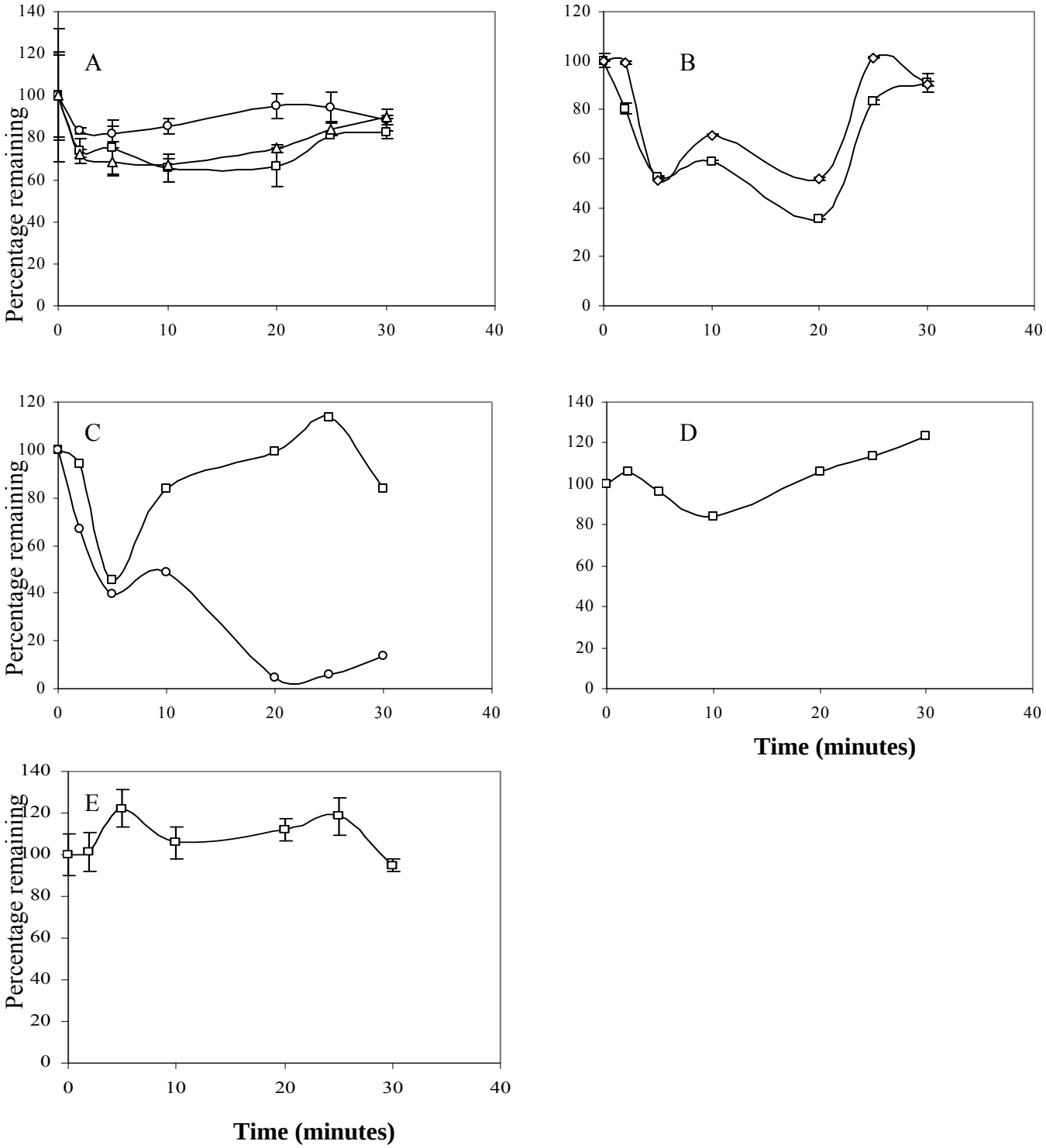

Figure 4.4.1: Percentage of initial intracellular concentrations of glycerol (□), arabitol (◊), mannitol (Δ) and trehalose (○) remaining when (A) *C. laurentii* US 1F, (B) *C. neoformans* US I1, (C) *F. floriforme* CBS 6240, (D) *R. mucilaginosa* CBS 5951, and (E) *C. macerans* CBS 2206T were subjected to hypo-osmotic shock from 0.972 a_w (0.86 M NaCl) to 0.996 a_w (0.09 M NaCl) in glucose-YNB. Bars represent the standard deviation of at least triplicate measurements. Data points C and D represent single measurement.

4.5 Survival of yeasts in soil cultures at different field capacity

The ability of yeasts to grow and survive in soil wetted to 100%, 25%, 10% and 5% of field capacity is illustrated in Figures 4.5.1 and 4.5.2. Generally, all the yeast strains that were tested were able to grow in the soil, particularly at 100% field capacity. However, viable cell yeast counts at this moisture content did seem to decline after 7 days for soils inoculated with *F. floriforme* CBS 6240 (Fig. 4.5.1E), *F. neoformans* CBS 0132 (Fig. 4.5.1F), *C. laurentii* CBS 0139 (Fig. 4.5.2A) and *F. capsuligeum* CBS 4381 (Fig. 4.5.2C). *R. mucilaginosa* CBS 5951 (Fig. 4.5.1B), *F. floriforme* CBS 6240 (Fig. 4.5.1E) and *F. capsuligeum* CBS 4381 (Fig. 4.5.2C) also showed growth in soil at 25% field capacity. However, strains *C. neoformans* US I1 (Fig. 4.5.1A), *C. laurentii* US 1F (Fig. 4.5.1C), *C. macerans* CBS 2206T (Fig. 4.5.1D) and *C laurentii* CBS 0139 (Fig. 4.5.2A) did not grow at this moisture content but survived up until the end of the experimental period. At lower soil moisture content (5% and 10% field capacity), the yeast strains either showed survival or decreased viability towards the end of the experimental period. Strain *C. neoformans* US I1 (Fig. 4.5.1A), *C. laurentii* US 1F (Fig. 4.5.1C), *C. macerans* CBS 2206T (Fig. 4.5.1D) and *R. mucilaginosa* CBS 5951 (Fig. 4.5.1B) and *F. floriforme* CBS 6240 (Fig. 4.5.1E) showed survival at both 5% and 10% field capacity. However, strain *F. neoformans* CBS 0132, *C. laurentii* CBS 0139 (Fig. 4.5.2A), *F. capsuligenum* CBS 4381 (Fig. 4.5.2C) and *S. salmonicolor* CBS 5937 (Fig. 4.5.2B) showed the decreased in viability either after 2 or 5 days. The viability of *C. laurentii* CBS 0139 (Fig. 4.5.2A) and *S. salmonicolor* CBS 5937 (Fig. 4.5.2B) decreased after 7 and 10 days of incubation.

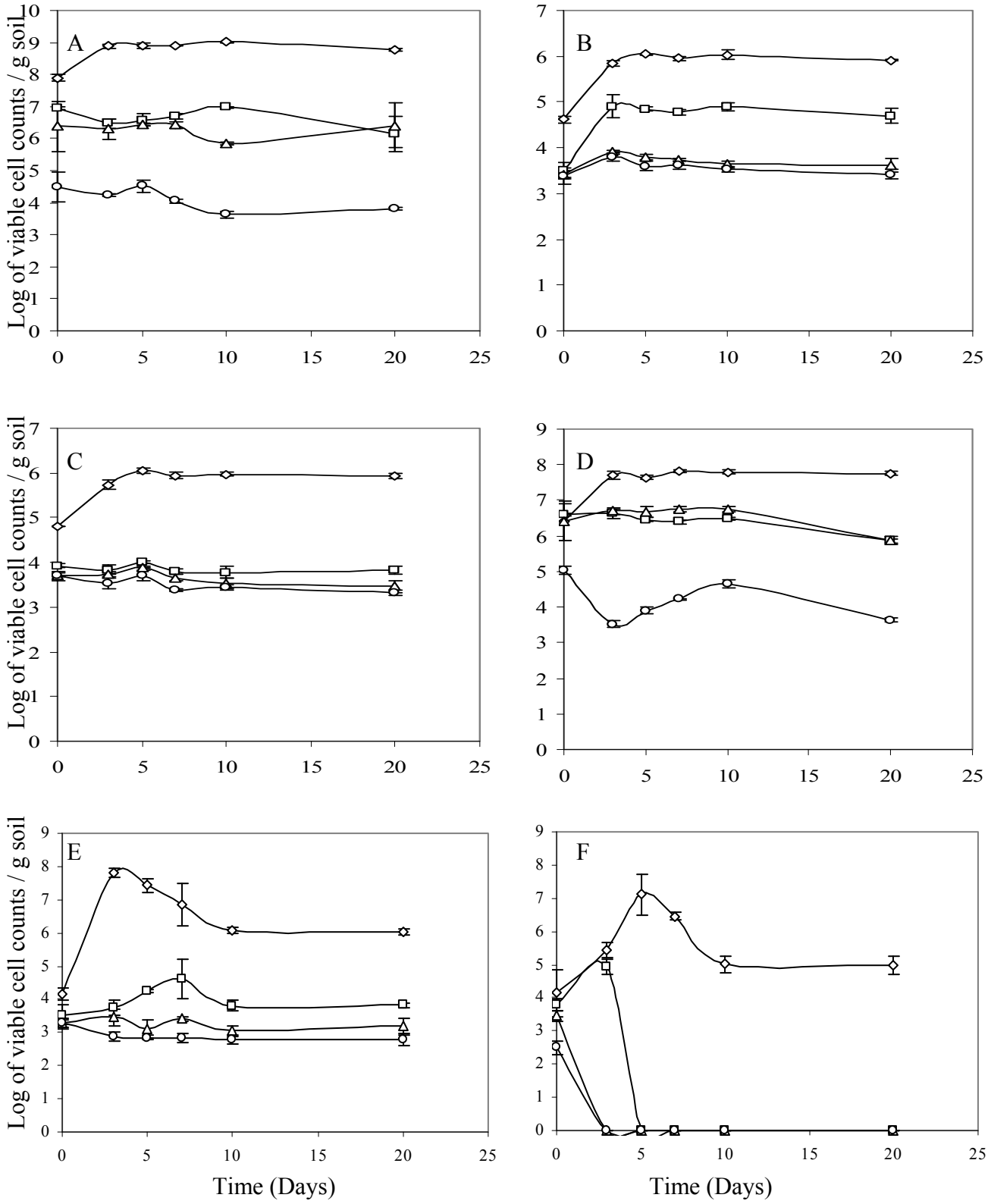

Figure 4.5.1: Survival of (A) *C. neoformans* US I1, (B) *R. mucilaginosa* CBS 5951, (C) *C. laurentii* 1F, (D) *C. macerans* CBS 2206T, (E) *F. floriforme* CBS 6240 and (F) *F. neoformans* CBS 0132 within soil differing in moisture content 100 % (◊), 25 % (□), 10 % (Δ) and 5 % (○). Data points represent the mean of triplicate determination and bars represent the standard deviation of the data.

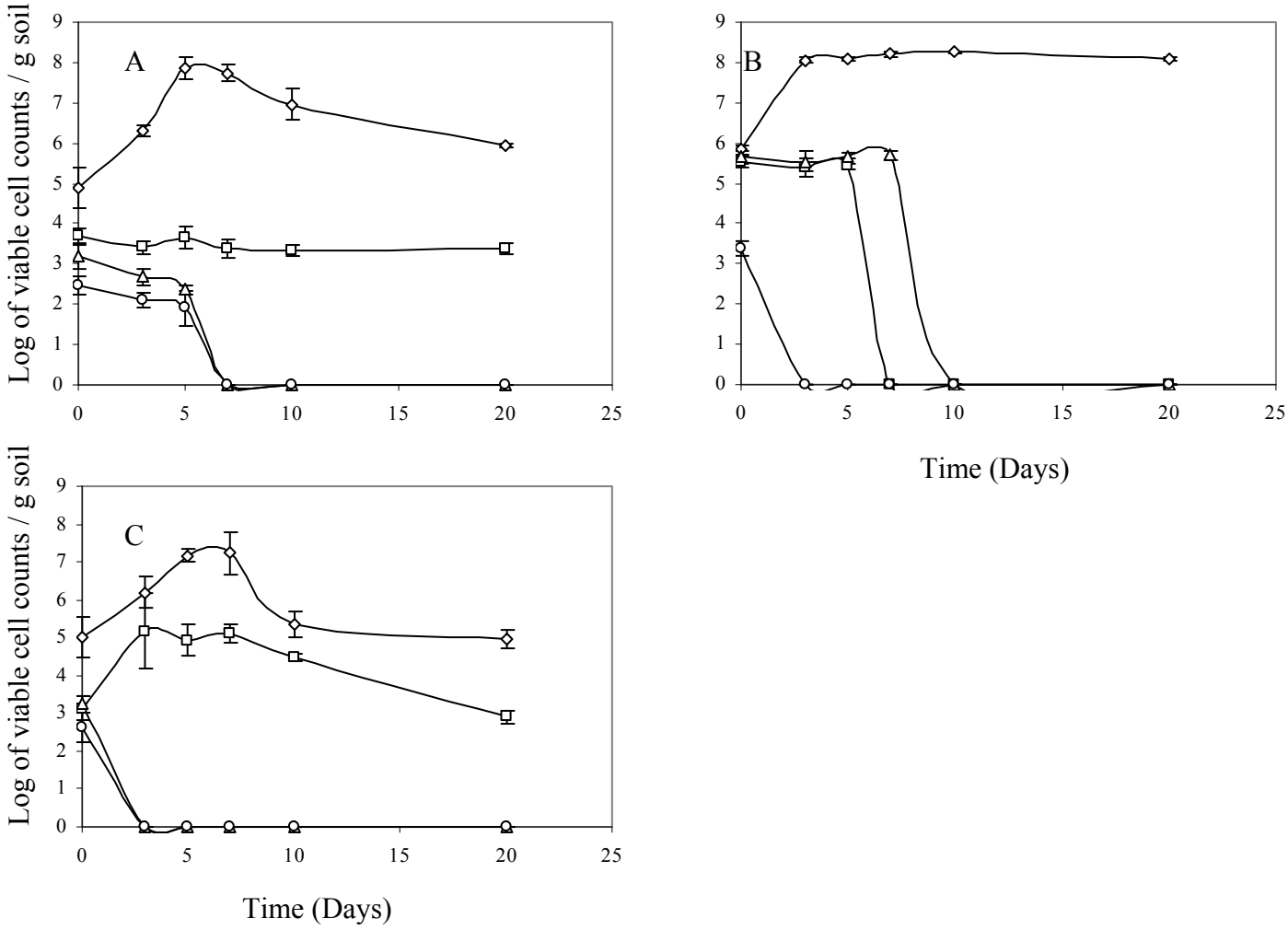

Figure 4.5.2: Survival of (A) *C. laurentii* CBS 0139, (B) *S. salmonicolor* CBS 5937 and (C) *F. capsuligenum* CBS 4381, within soil differing in moisture content 100 % (◊), 25 % (□), 10 % (Δ) and 5 % (○). Data points represent the mean of triplicate determination and bars represent the standard deviation of the data.

5. Discussion

Comparison of minimum growth a_w of ascomycetous with basidiomycetous yeasts

The values of the minimum a_w for the 41 strains of basidiomycetous yeasts in this study are in the contrast to the minimum a_w values of ascomyceotous yeast disclosed by van Eck et al. (1993). For example, no strain of basidiomycetous yeast was found to grow below 0.9 a_w either when subjected to NaCl or sorbitol. However, the minimum a_w values for the growth for a number of species of ascomycetous yeast were previously reported to be less than 0.9 when a_w adjusted with glucose or NaCl. Fourteen ascomycetous yeast species had minimum growth a_w (NaCl) less than 0.9 and 19 species had a minimum growth a_w (glucose) less than 0.9, with *Z. rouxii* the most extreme at 0.65 a_w (glucose) (van Eck et al., 1993). In all instances the minimum growth a_w of basidiomycetous yeasts was lower when adjusted with NaCl than sorbitol, except only *F. floriforme* CBS 6240, which had a minimum growth a_w of 0.925 and 0.90 when adjusted respectively with NaCl and sorbitol. This observation suggests that basidiomycetous yeasts are mainly salt tolerant. This is in contrast to ascomycetous yeasts, where more strains of ascomycetous yeasts evaluated were glucose tolerant than salt tolerant (van Eck et al., 1993). Furthermore, most yeast isolated from high sugar foods have been found to belong to the ascomycetous group (Tokuoka et al., 1985; Jeremi et al., 1987; Tokuoka, 1993). The greater tolerance of basidiomycetous yeasts to NaCl than to sorbitol might be as a result of the physico-chemical composition of their natural habitat. For example, ascomycetous yeasts are the dominant yeasts in environments with high concentrations of sugar (Tokuoka, 1993; van Eck et al., 1993), whereas basidiomycetous yeasts seem to dominate habitats such as soil, plant surfaces and bird droppings (Botha, 2006).

Comparison of osmolytes accumulated by ascomyetous with basidiomycetous yeasts

This study has shown the importance of osmolytes identified by the ^{13}C NMR spectroscopy in the osmoregulation of basidiomycetous yeasts. These yeasts accumulated glycerol, mannitol, arabitol and trehalose intracellularly when exposed to reduced a_w (0.95 a_w). Glycerol, arabitol and mannitol are known to be the main solutes accumulated in yeasts when subjected to osmotic stress (Spencer and Spencer, 1978). As is found in ascomycetous yeasts (Reed et al., 1987; van Eck et al., 1993), glycerol was present when basidiomycetous yeast was grown at reduced a_w (0.95 a_w) and these yeast appeared

to regulate glycerol as the main osmolyte. Little attention has focused on the osmolytes accumulated by basidiomycetous fungi under osmotic stress but Jennings (1984) found that the three basidiomycetous fungi investigated were able to produce most of the osmolytes observed in this study.

Some strains of basidiomycetous yeasts, namely *C. curvatus* CBS 2176 (Fig. 4.2.2B), *F. capsuligenum* CBS 4381 (Fig. 4.2.6A), *F. capsuligenum* CBS 6122.2 (Fig. 4.2.6B), *F. floriforme* CBS 6240 (Fig. 4.2.6C) and *C. laurentii* US 1F (Fig. 4.2.3A), accumulated trehalose intracellularly as an additional osmolyte when a_w was reduced to $0.95a_w$. The role of trehalose in osmoregulation is less clear but Hounsa et al. (1998) noted that trehalose appeared to be important in the survival of *S. cerevisiae*. For example, *S. cerevisiae* with high level of trehalose shows high resistance to dehydration (Hounsa et al., 1998). Generally, trehalose is a storage carbohydrate and accumulates in the cells during early stationary phase (Hounsa et al., 1998). Furthermore, the work of Hounsa et al, (1998) indicated that trehalose does not act at any stage as a reserve for glycerol synthesis when exposed to osmotic stress.

The accumulation of intracellular solutes in basidiomycetous yeasts in response to osmotic stress is inconsistent. For example, *C. laurentii* US 1F is the only strain that accumulated three intracellular osmolytes when exposed to reduced a_w (0.95 a_w) whereas the other basidiomycetous yeasts accumulated either two osmolytes or just glycerol. This observation is similar to ascomycetous yeasts, where *P. sorbitophila* was found to accumulate glycerol, arabitol and erythritol (Kayingo et al., 2001) whereas all the other yeasts studied by van Eck et al. (1993) were found to accumulate arabitol and mannitol in addition to glycerol or just glycerol, as in *S. cerevisiae*. Interestingly, eleven strains (Table 4.2) of basidiomyceotus yeasts accumulated only glycerol as intracellular solute. The role of the secondary polyol in osmoregulation is less clear. Brown et al., (1986) suggested that the secondary solutes may play a role of sustaining yeast viability when exposed to osmotic shock. Interestingly, the three basidiomycetous yeasts evaluated accumulated osmolytes consistently over time from early exponential phase until the late stationary phase (Figures 4.3.1-3).

Release of osmolytes from yeasts exposed to hypo-osmotic shock

When yeasts cells were exposed to hypo-osmotic shock only 6 strains (Table 4.3) showed loss of viability when exposed to hypo-osmotic shock. The other strains survived the osmotic shock when the osmotic stress was removed (from 0.972 a_w to 0.998a_w). Only two of the five species were found to rapidly release their osmolytes upon hypo-osmotic stress (Figure 4.4.1). *F. floriforme* CBS 6240 (Fig. 4.4.1C) and *C. neoformans* US I1 (Fig. 4.4.1B), released approximately 45% of the intracellular glycerol in 5 min when exposed to hypo-osmotic shock. However, the other three strains were reluctant to release their osmolytes and other mechanisms might be used to respond to hypo-osmotic stress. Luyten et al. (1995) found that the significant loss of viability by *S. cerevisiae* after hypo-osmotic shock occurred once a gene (*FPS1*) encoding a MIP channel protein (also known as a glycerol facilitator) was deleted due to ineffective osmolyte export. Tang et al. (2005) reported that the ascomycete *Z. rouxii* behaved similarly during hypo-osmotic stress by releasing glycerol and arabitol by a MIP channel protein. Furthermore, the ascomycetous yeast *P. sorbitophila* was able to rapidly release glycerol, arabitol and erythritol upon hypo-osmotic stress but the channel involved was not characterized (Kayingo et al., 2001). No data has been reported in literature on whether basidiomycetous yeasts release osmolytes using similar export channels than ascomycetous yeasts.

Survival of yeasts in soil cultures at different field capacity

From the results obtained (Fig.4.5.1 and 4.5.2) it is obvious that the yeasts showed both inter and intraspecific variation in their response to soil with a lower moisture content. Taking into account the ability to grow at 25% field capacity and to survive at 5 and 10% field capacity, it can be concluded that the yeast strains that were most resistant towards lower moisture levels in the soil were *R. mucilaginosa* CBS 5951 and *F. floriforme* CBS 6240. The former species has been found in a wide diversity of habitats that includes soil, plant materials as well as animal materials while the latter species was isolated from the same diversity of habitats that include wood, terrestrial and marine environments (Fell, 1976; Spencer and Spencer, 1997). The yeasts that seemed to be the least tolerant towards lower moisture levels in soil, were *S. salmonicolor* CBS 5937 and *F. neoformans* CBS 0132. Both species are associated with decomposing vegetative debris and are mostly found on leaf surfaces (Fell, 1976; Spencer and Spencer, 1997), but the latter is also a well known opportunistic mammalian pathogen (Fell et al., 2001). Interestingly, no relationship could be found between the type and number of intracellular osmolytes accumulated (Table 4.2) when exposed to osmotic stress (0.95a_w NaCl) and

the ability to grow and survive in soil with a lower moisture content. Similarly, the ability of the yeasts to grow and survive in soil with a lower moisture content did not correlate with their minimum a_w for growth in a liquid media (Table 4.1). Therefore, it seems that growth and survival of the yeasts within soil with a low moisture content do not solely depend on the type of intracellular osmolytes produced by the yeast or its ability to survive osmotic stress in liquid medium. Other factors, such as the physio-chemical composition of the soil, e.g. nutrient and heavy metal concentrations, as well as soil pH (Cornelissen et al. 2003; Botha, 2006) may also play a role in the survival of a particular yeast species in soil.

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Chapter.7. Appendices

7.1. Appendix 1. Growth of basidiomycetous yeasts at various a_w (NaCl and sorbitol)

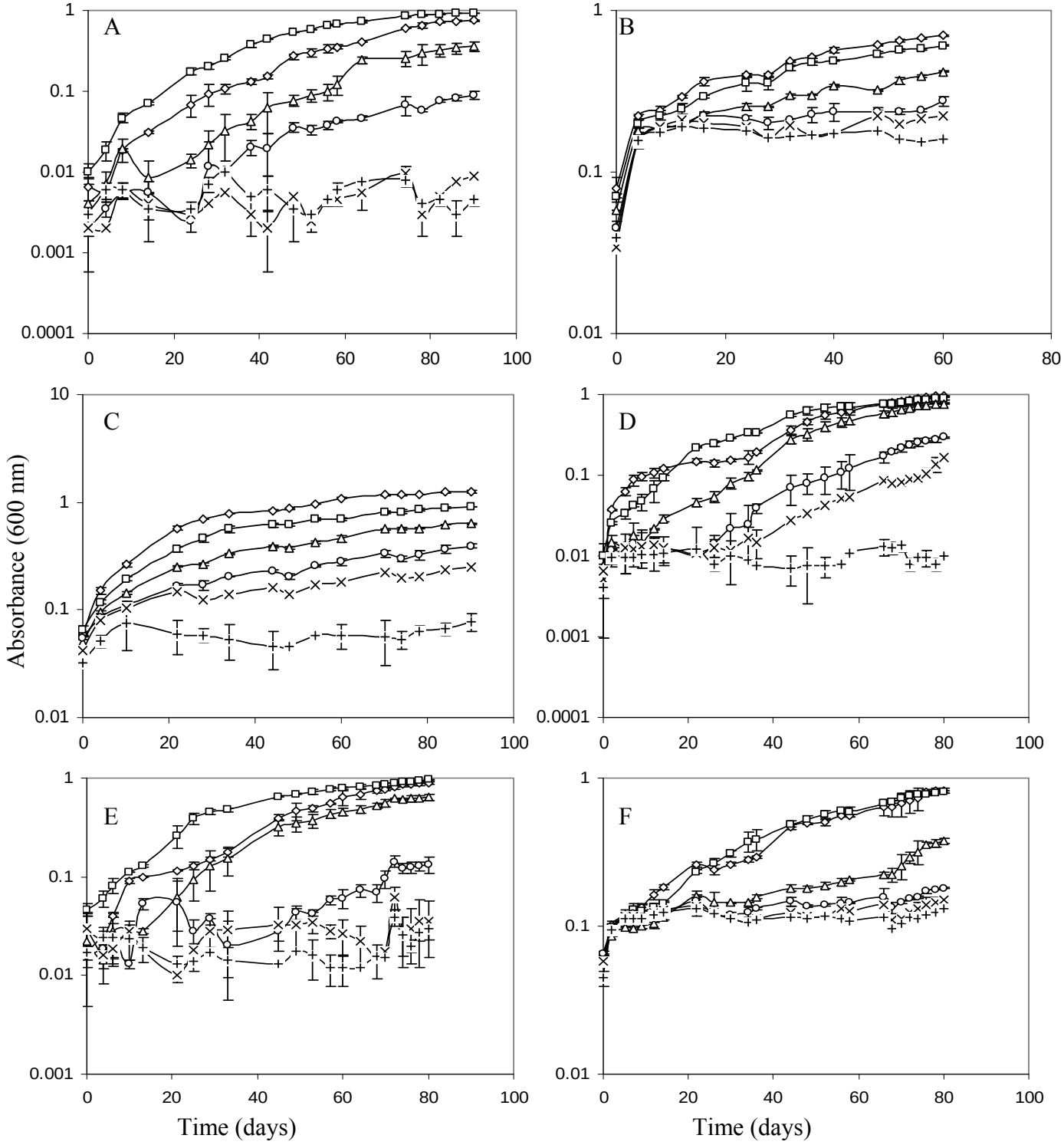

Figure 7.1.1: Growth of (A) *B. albus* CBS 6097 (B) *B. dendrophila* CBS 6074T, (C) *C. albidus* CBS 5737, (D) *C. amylolentus* CBS 6039T, (E) *C. bhutanensis* CBS 6294T (F) *C. curvatus* CBS 2176 at 0.998 a_w (◊), 0.975 a_w (□), 0.95 a_w (Δ), 0.925 a_w (○) 0.90 a_w (X) and (+) 0.875 a_w (NaCl).

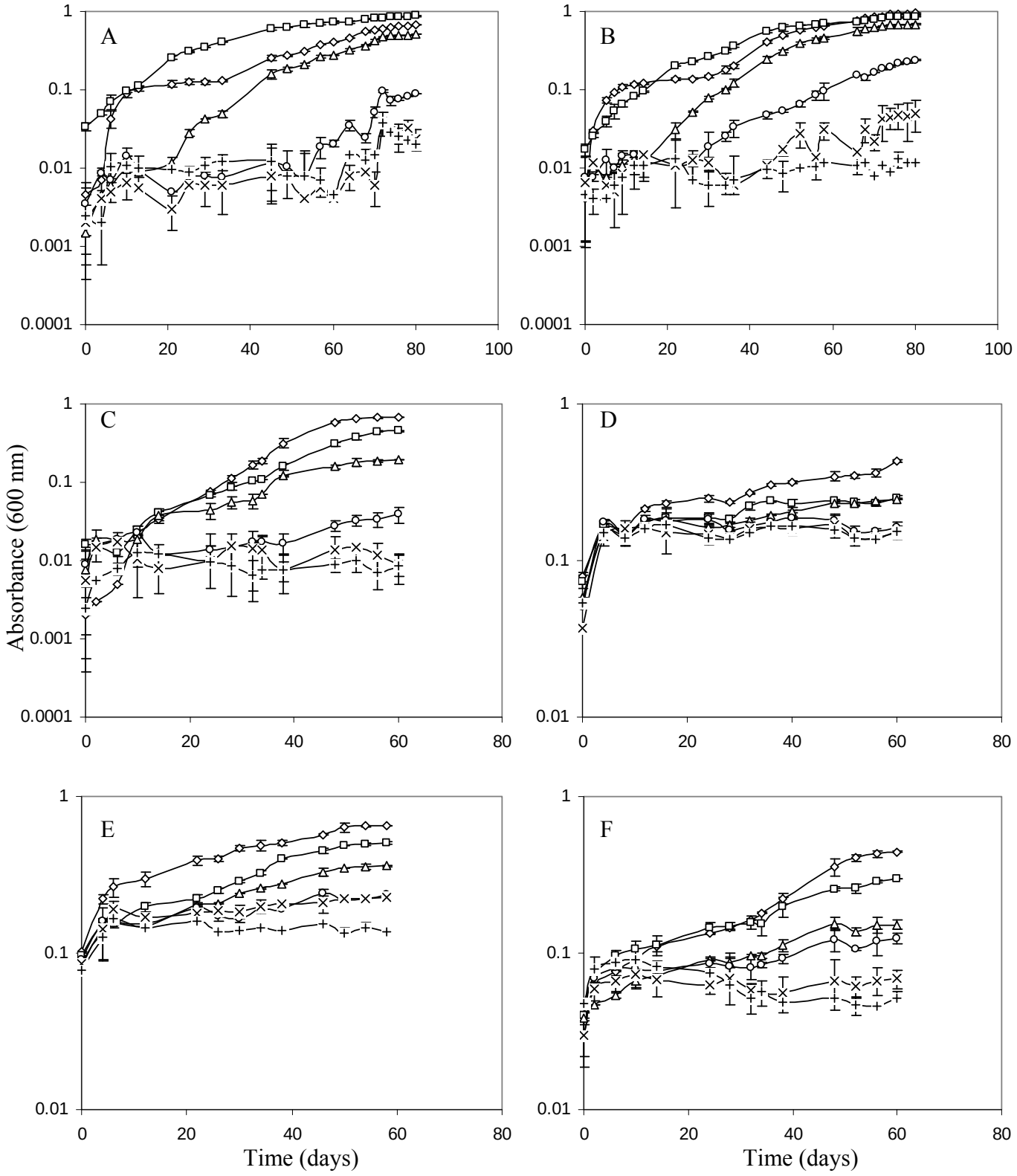

Figure 7.1.2: Growth of (A) *C. gastricus* CBS 1927, (B) *C. hungaricus* CBS 5124, (C) *C. laurentii* US 1A, (D) *C. laurentii* US 1F, (E) *C. macerans* CBS 2206T, (F) *C. neoformans* US 132T at 0.998 a_w (◊), 0.975 a_w (□), 0.95 a_w (Δ), 0.925 a_w (○) 0.90 a_w (X) and (+) 0.875 a_w (NaCl).

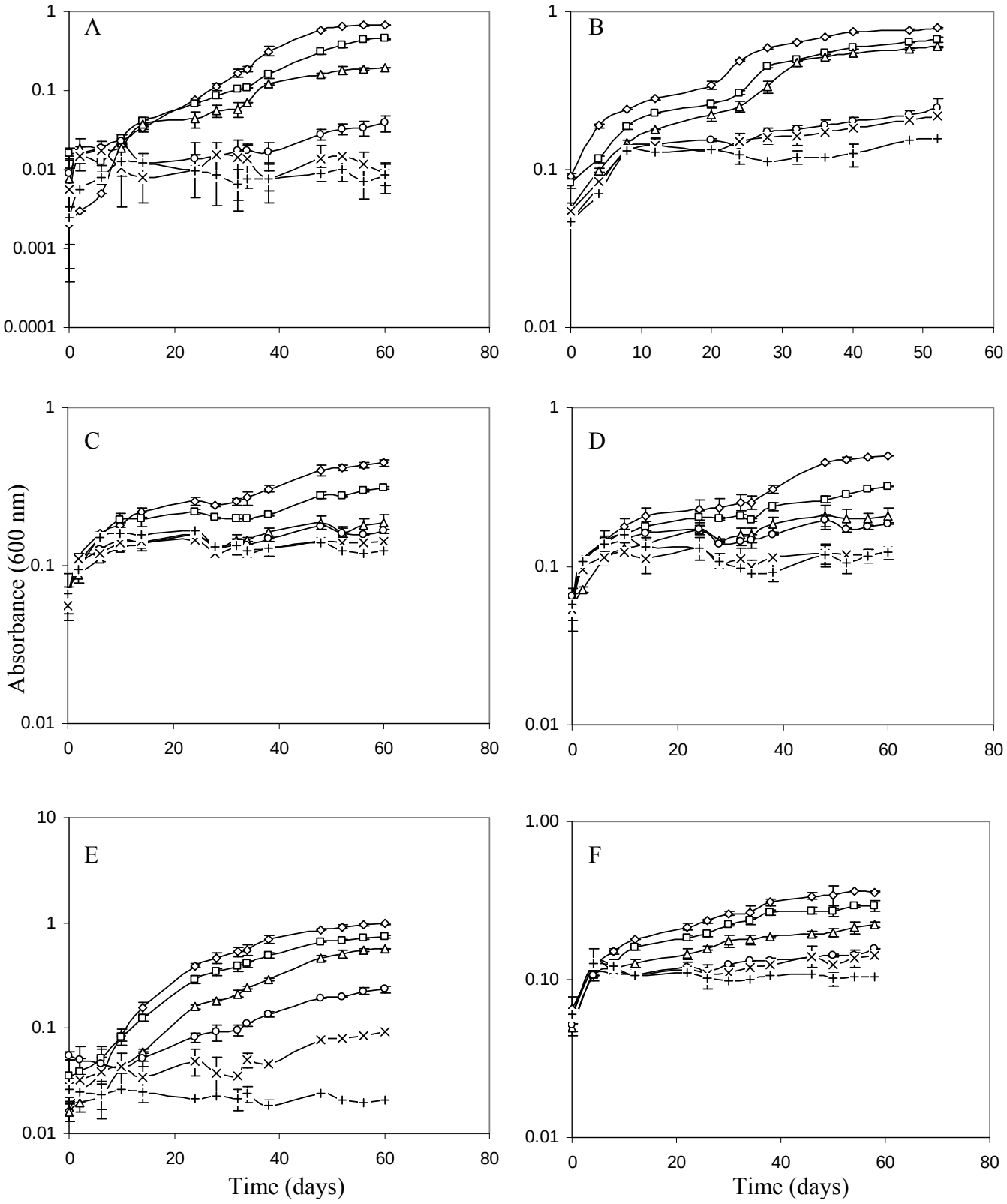

Figure 7.1.3: Growth of (A) *C. neoformans* US C2, (B) *C. neoformans* US I1, (C) *C. neoformans* US I5, (D) *C. neoformans* US I6, (E) *C. neoformans* US S5, (F) *C. neoformans* US I4 at 0.998 a_w (◊), 0.975 a_w (□), 0.95 a_w (Δ), 0.925 a_w (○) 0.90 a_w (X) and (+) 0.875 a_w (NaCl).

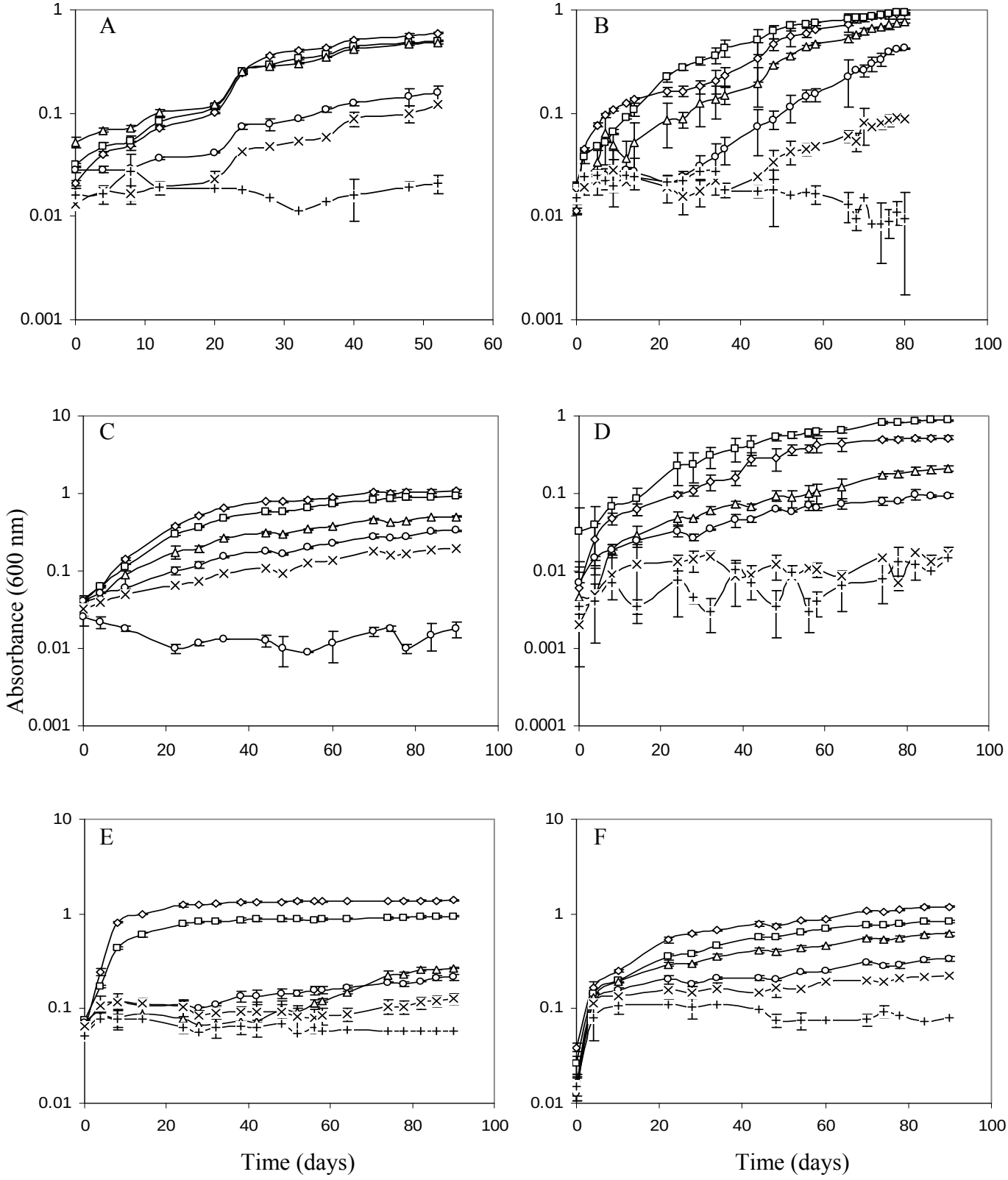

Figure 7.1.4: Growth of (A) *C. podzolicus* US 5A, (B) *C. terreus* CBS 1895T, (C) *C. laurentii* CBS 0139, (D) *F. capsuligenum* CBS 4381, (E) *F. floriforme* CBS 6240 and (F) *F. neoformans* CBS 0132 at 0.998 a_w (◊), 0.975 a_w (□), 0.95 a_w (Δ), 0.925 a_w (○) 0.90 a_w (X) and (+) 0.875 a_w (NaCl).

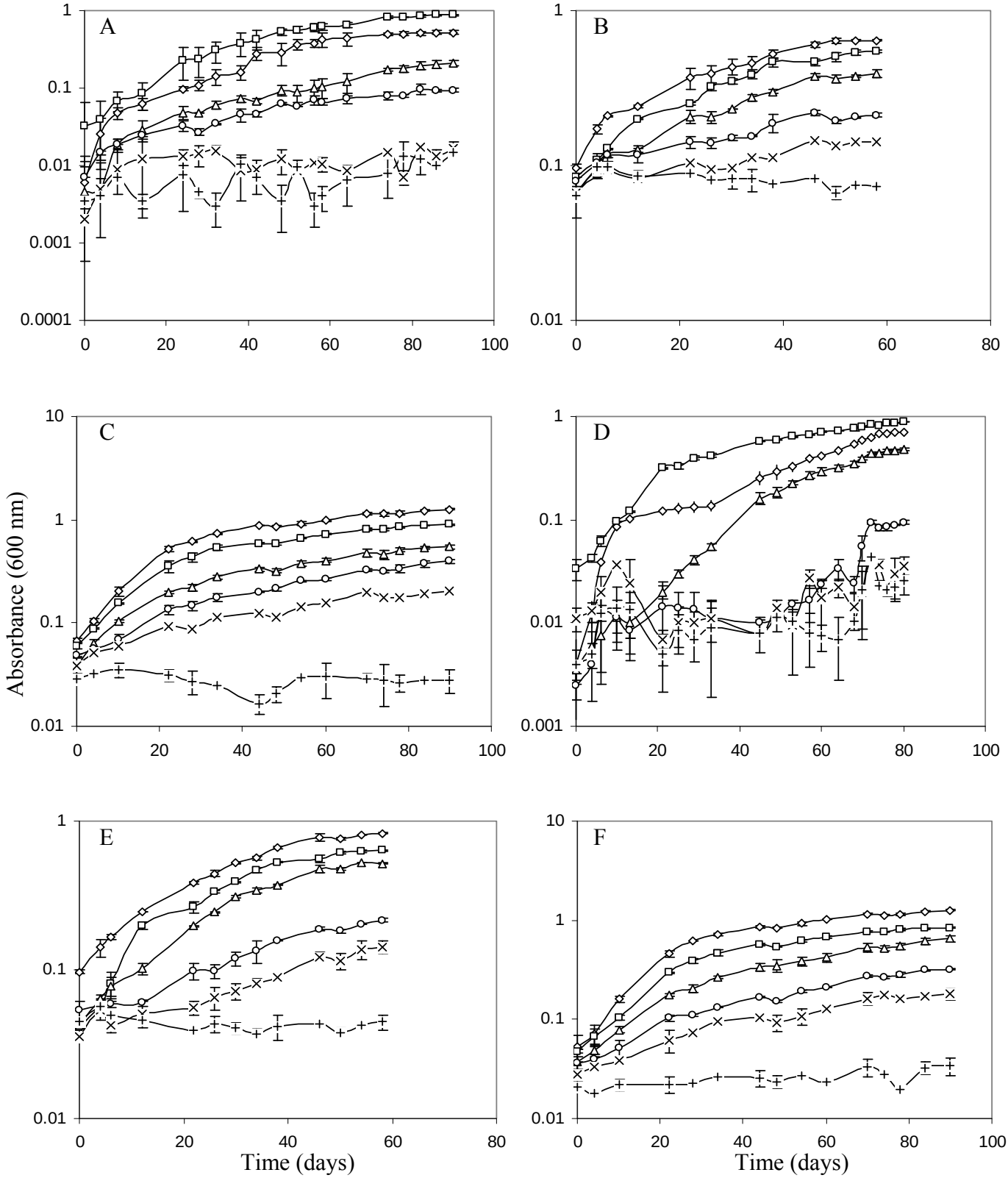

Figure 7.1.5: Growth of (A) *F. neoformans* CBS 0884, (B) *F. neoformans* CBS 6885, (C) *F. unigetulattum* CBS 2770, (D) *R. araucariae* CBS 6031T, (E) *R. glutinis* CBS 0020 and (F) *R. graminis* CBS 2826 at 0.998 a_w (◊), 0.975 a_w (□), 0.95 a_w (Δ), 0.925 a_w (○) 0.90 a_w (X) and (+) 0.875 a_w (NaCl).

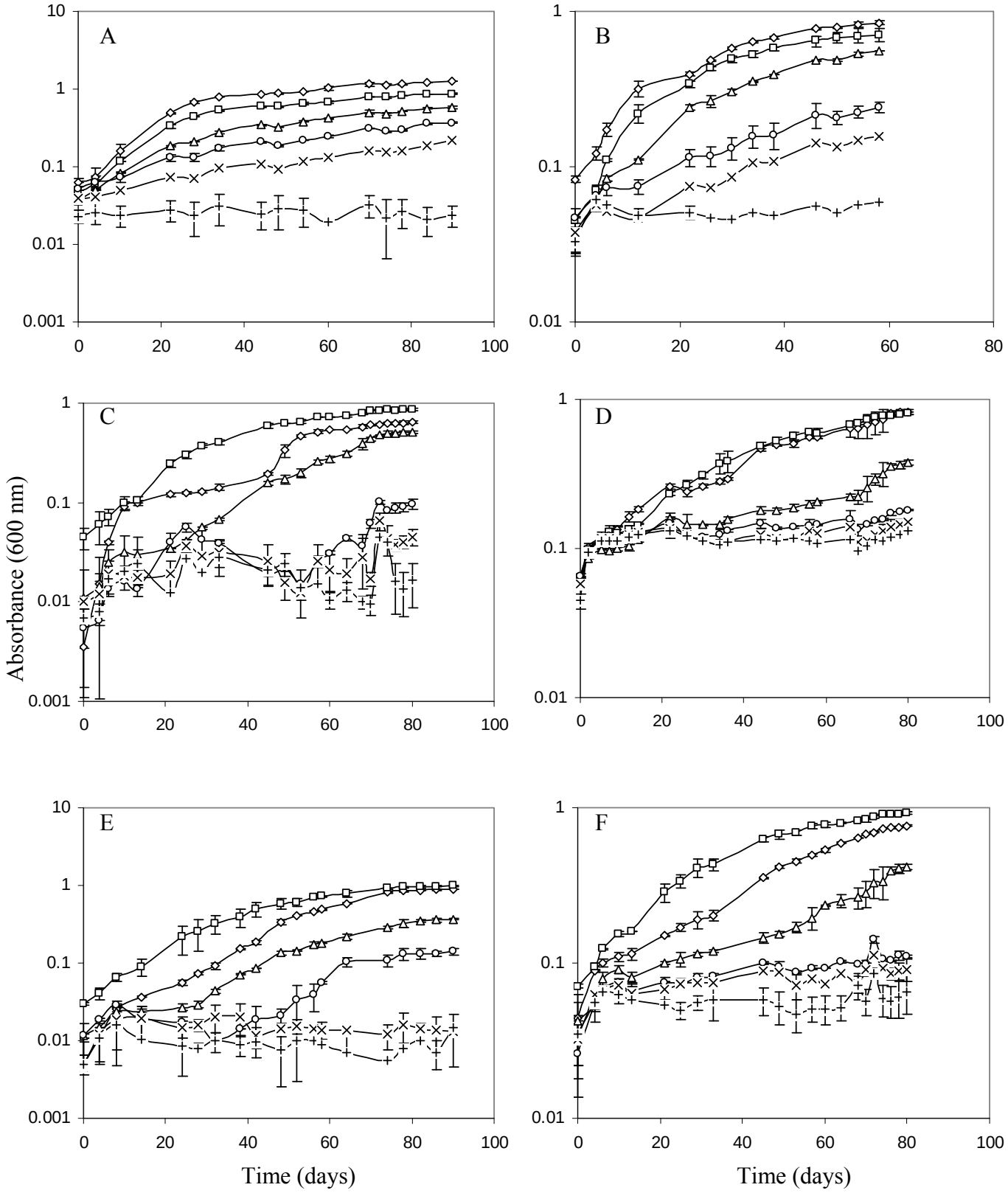

Figure 7.1.6: Growth of (A) *R. lactosa* CBS 5856T, (B) *R. minuta* var *minuta* CBS 2172, (C) *R. minuta* var *minuta* CBS 2177, (D) *R. mucilaginosa* CBS 5951, (E) *R. toruloides* CBS 0349 and (F) *Rhodotorula sp* CBS 5143 at 0.998 a_w (◊), 0.975 a_w (□), 0.95 a_w (Δ), 0.925 a_w (○) 0.90 a_w (X) and (+) 0.875 a_w (NaCl).

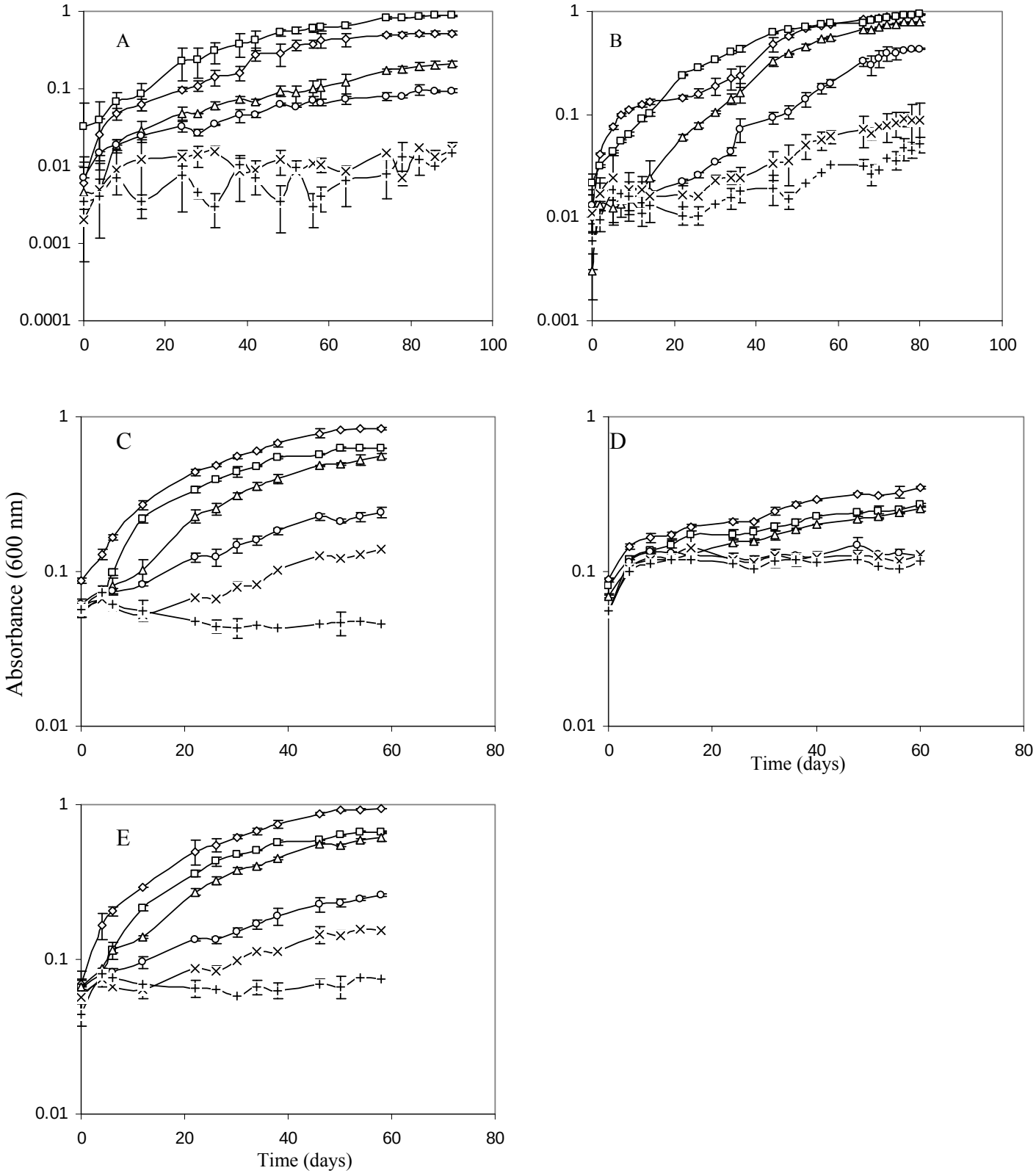

Figure 7.1.7: Growth of (A) *F. capsuligenum* CBS 6122.2 (B) *S. halophilus* CBS5 5628Y, (C) *S. halophilus* CBS 4609Y, (D) *S. salmonicolar* CBS 5937, and (E) *T. cuteneum var cuteneum* CBS 2466NT at 0.998 a_w (◊), 0.975 a_w (□), 0.95 a_w (Δ), 0.925 a_w (○) 0.90 a_w (X) and (+) 0.875 a_w (NaCl).

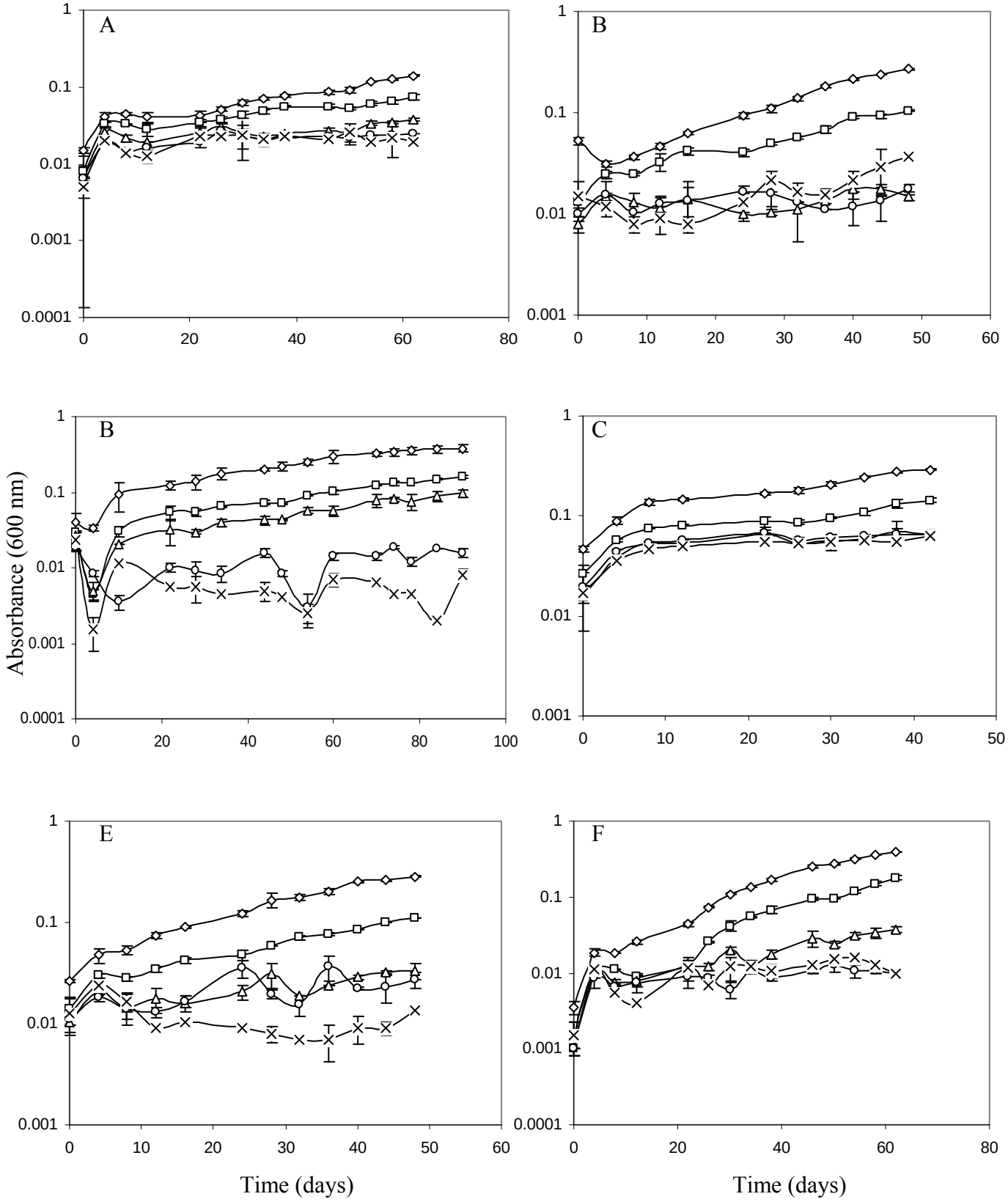

Figure 7.1.8: Growth of (A) *B. albus* CBS 6097, (B) *B. dendrophila* CBS 6074T, (C) *C. albidus* CBS 5737, (D) *C. amylolentus* CBS 6039T (E) *C. bhutanensis* CBS 6294T and (F) *C. curvetus* CBS 2176 at 0.975 a_w (◊), 0.95 a_w (□), 0.925 a_w (Δ), 0.90 a_w (○), and 0.875 a_w (X) in sorbitol.

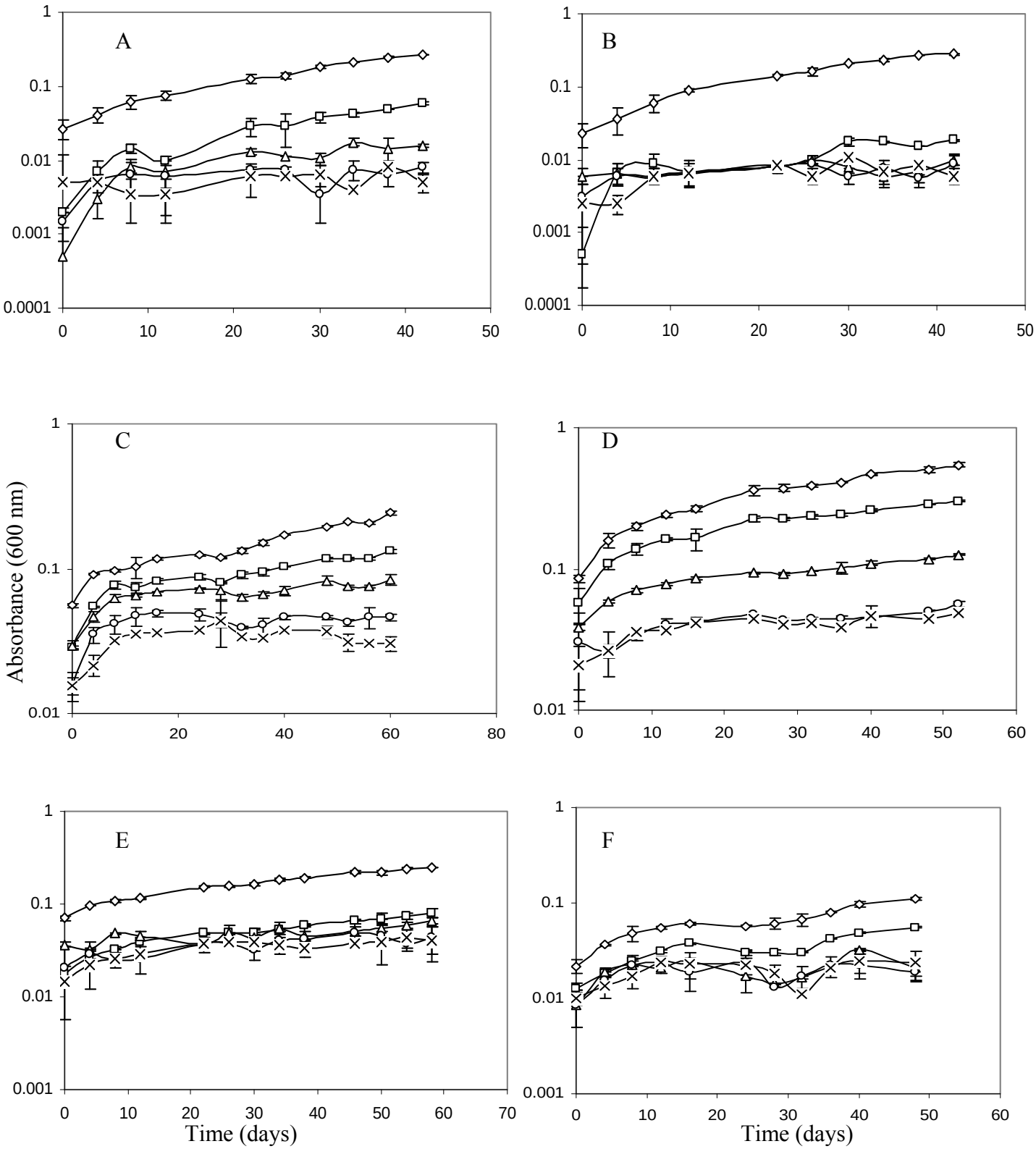

Figure 7.1.9: Growth of (A) *C. gastricus* CBS 1927, (B) *C.hungaricus* CBS 5124, (C) *C. laurentii* US 1A, (D) *C. laurentii* US 1F (E) *C. macerans* CBS 2206T and (F) *C. neoformans* US 132T at 0.975 a_w (◊), 0.95 a_w (□), 0.925 a_w (Δ), 0.90 a_w (○), and 0.875 a_w (X) in sorbitol.

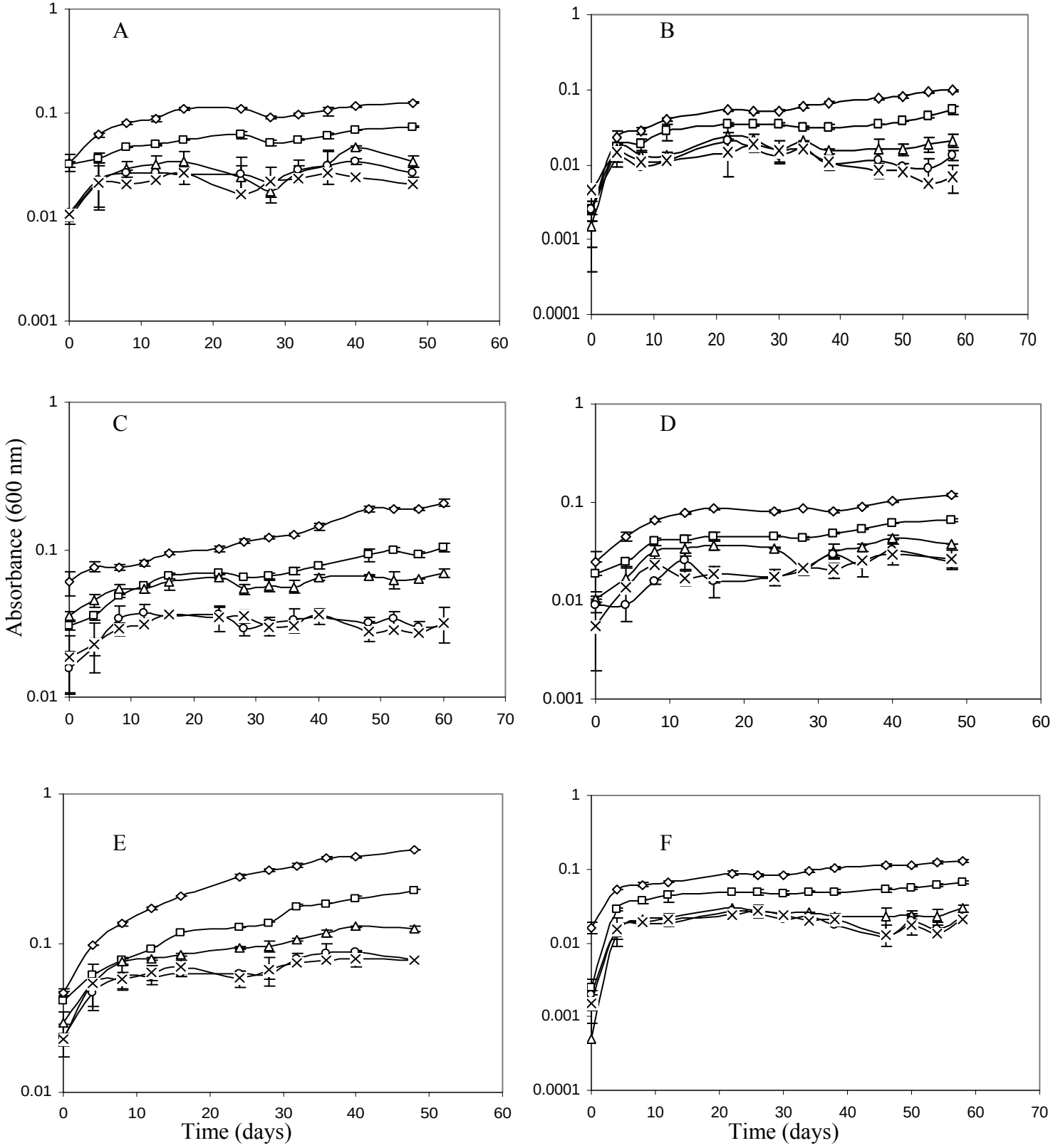

Figure 7.1.10: Growth of (A) *C. neoformans* US C2, (B) *C. neoformans* US I1, (C) *C. neoformans* US I5, (D) *C. neoformans* US 16 (E) *C. neoformans* US S5 and (F) *C. neoformans* US 14 at 0.975 a_w (◊), 0.95 a_w (□), 0.925 a_w (Δ), 0.90 a_w (○), and 0.875 a_w (X) in sorbitol.

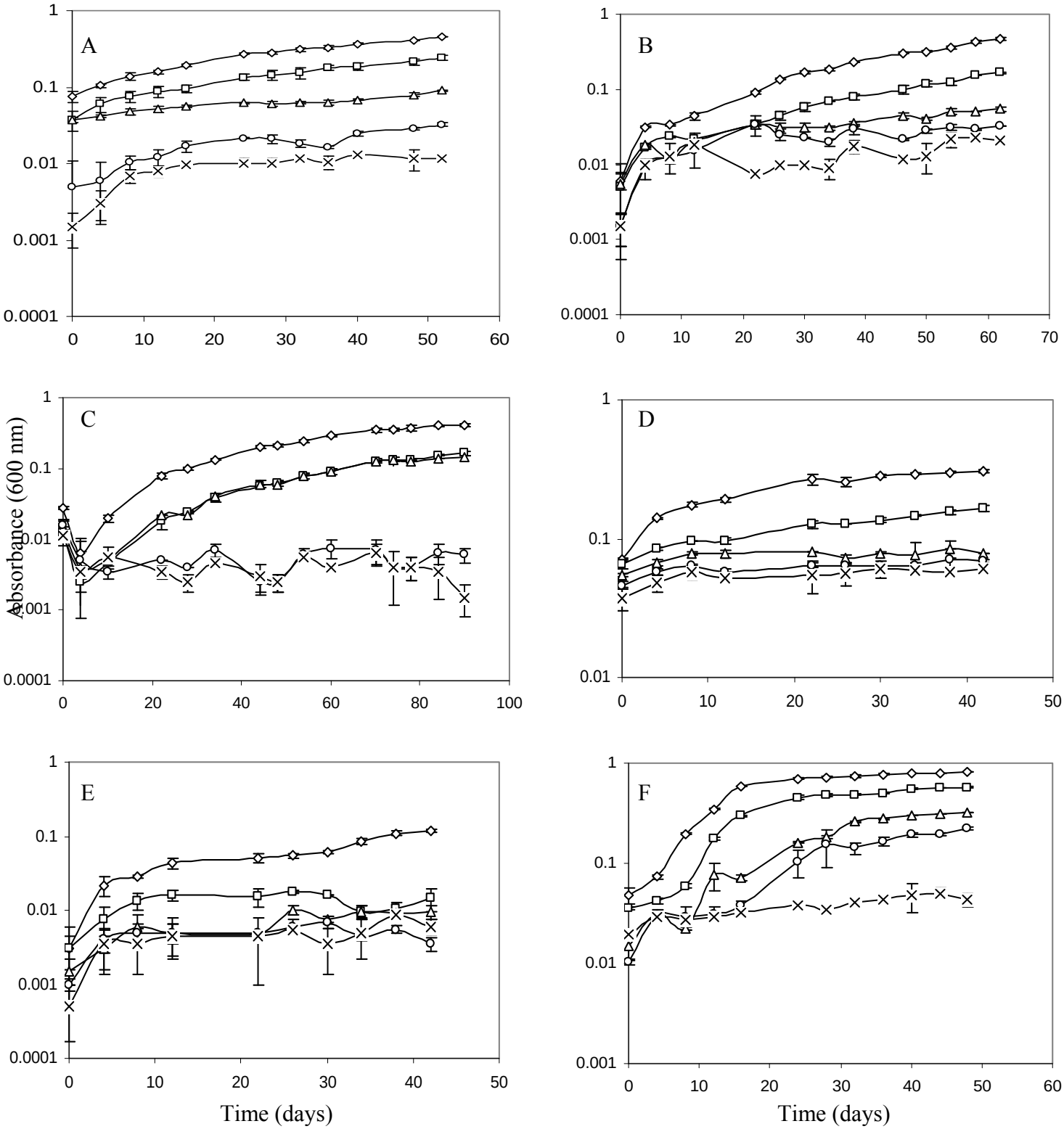

Figure 7.1.11: Growth of (A) *C. podzolicus* US 5A, (B) *C. terreus* CBS 1895T, (C) *C. laurentii* CBS 0139, (D*) F. capsuligenum* CBS 4381, (E) *C. capsuligenum* CBS 6122.2 and (F) *C. floriforme* 6240 at 0.975 a_w (◊), 0.95 a_w (□), 0.925 a_w (Δ), 0.90 a_w (○), and 0.875 a_w (X) in sorbitol.

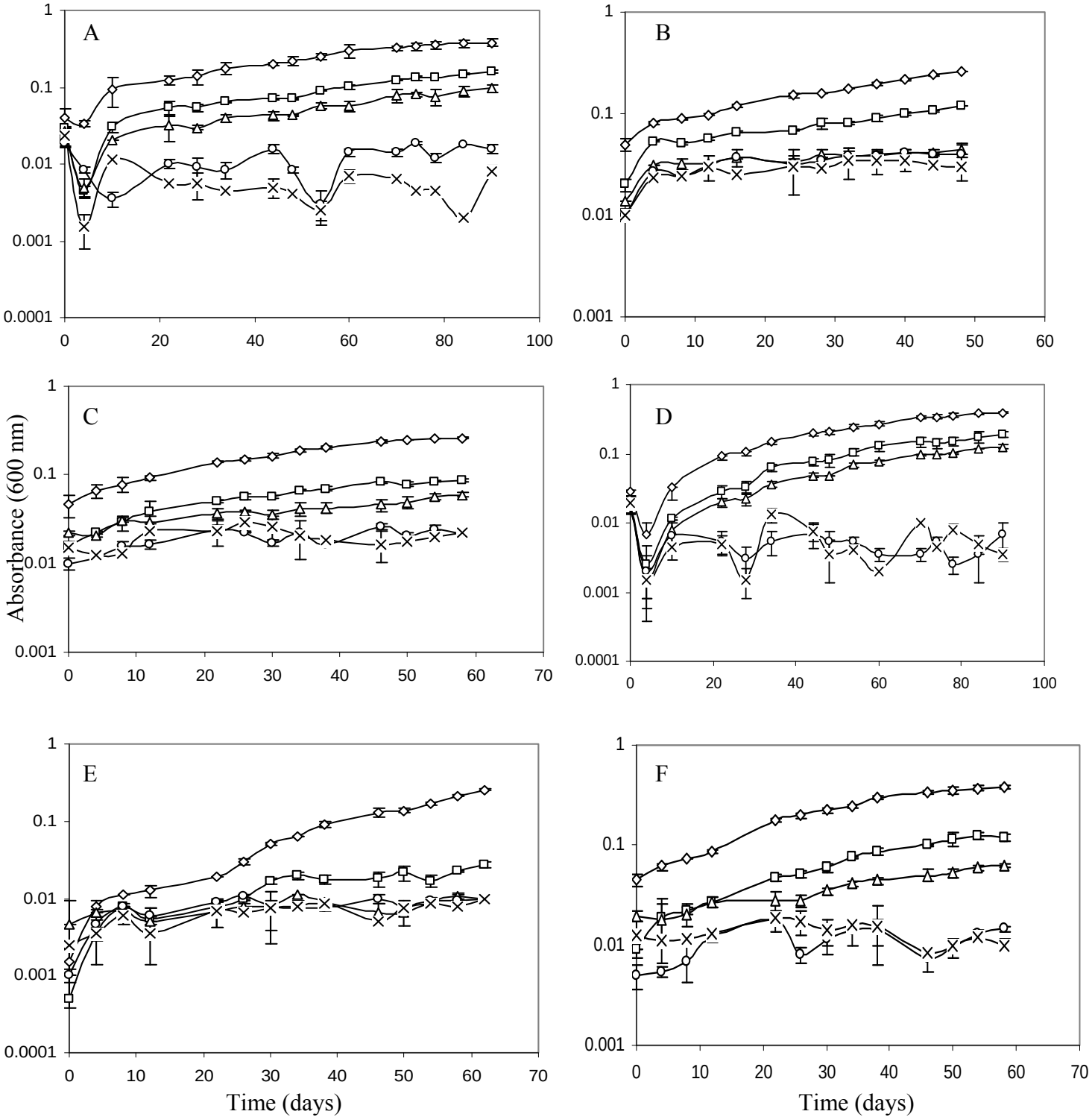

Figure 7.1.12: Growth of (A) *F. neoformans* CBS 0132, (B) *F. neoformans* CBS 0884, (C) *F. neoformans* CBS 6885, (D) *F. unigetulattum* CBS 2770, (E) *R. araucariae* CBS 6031T and (F) *R. glutinis* CBS 0020 at 0.975 a_w (◊), 0.95 a_w (□), 0.925 a_w (Δ), 0.90 a_w (○), and 0.875 a_w (X) in sorbitol.

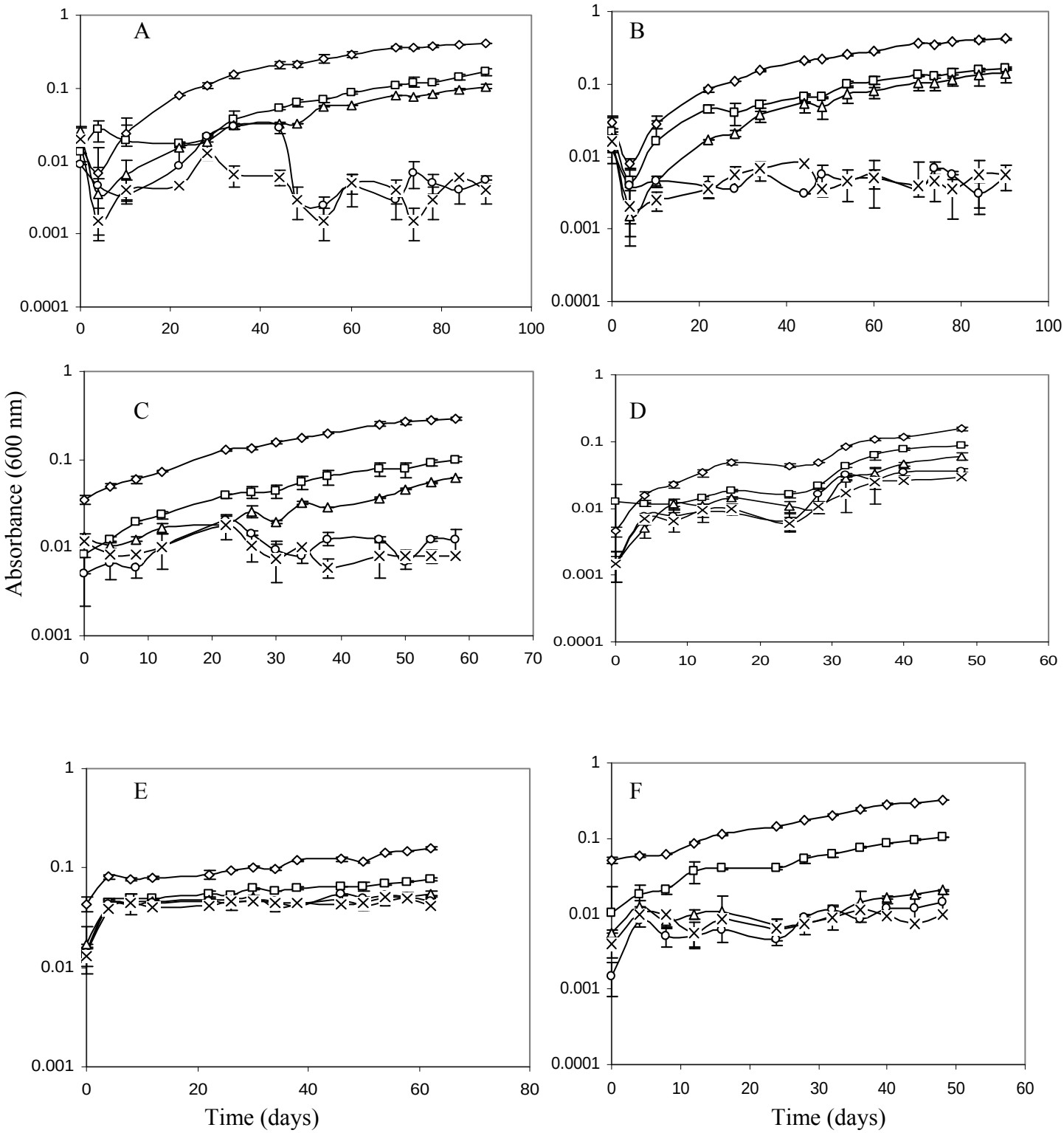

Figure 7.1.13: Growth of (A) *R. graminis* CBS 2826, (B) *R. lactosa* CBS 5826T, (C) *R. minuta* CBS 2172, (D) *R. minuta* CBS 2177, (E) *R. mucilaginosa* CBS 5951 and (F) *R. toruloides* CBS 0349 at 0.975 a_w (◊), 0.95 a_w (□), 0.925 a_w (Δ), 0.90 a_w (○), and 0.875 a_w (X) in sorbitol.

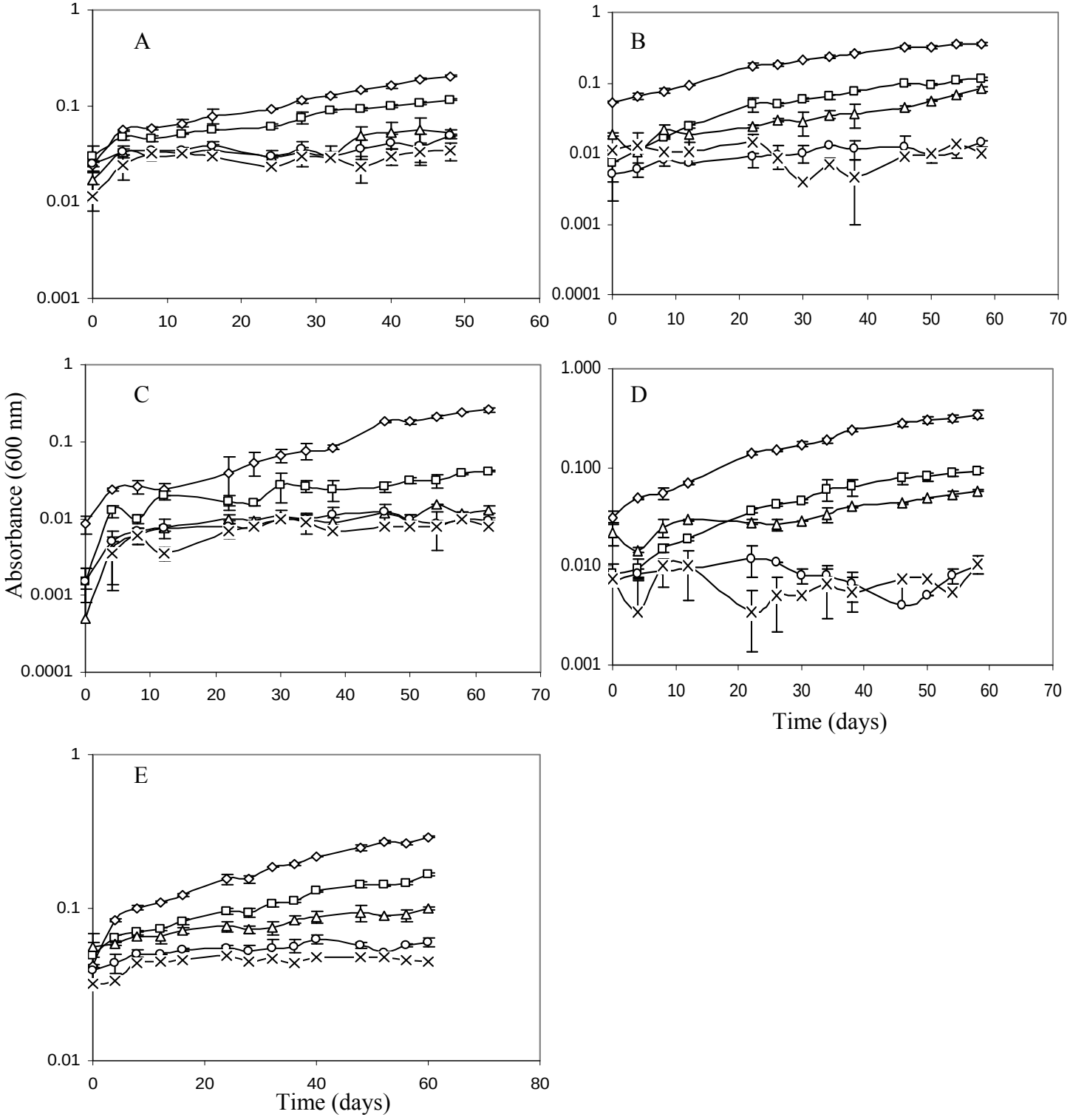

Figure 7.1.14: Growth of (A) *Rhodotorula sp* CBS 5143, (B) *S. halophilus* CBS 4609Y, (C) *S. halophilus* CBS 5826Y, (D) *S. salmonicolor* CBS 5937, and (E) *T. cuteneum* CBS 2466NT at 0.975 a_w (◊), 0.95 a_w (□), 0.925 a_w (Δ), 0.90 a_w (○), and 0.875 a_w (X) in sorbitol.

7.2. Appendix 2. ^{13}C NMR spectra of basidiomycetous yeasts grown in media with and without addition of NaCl

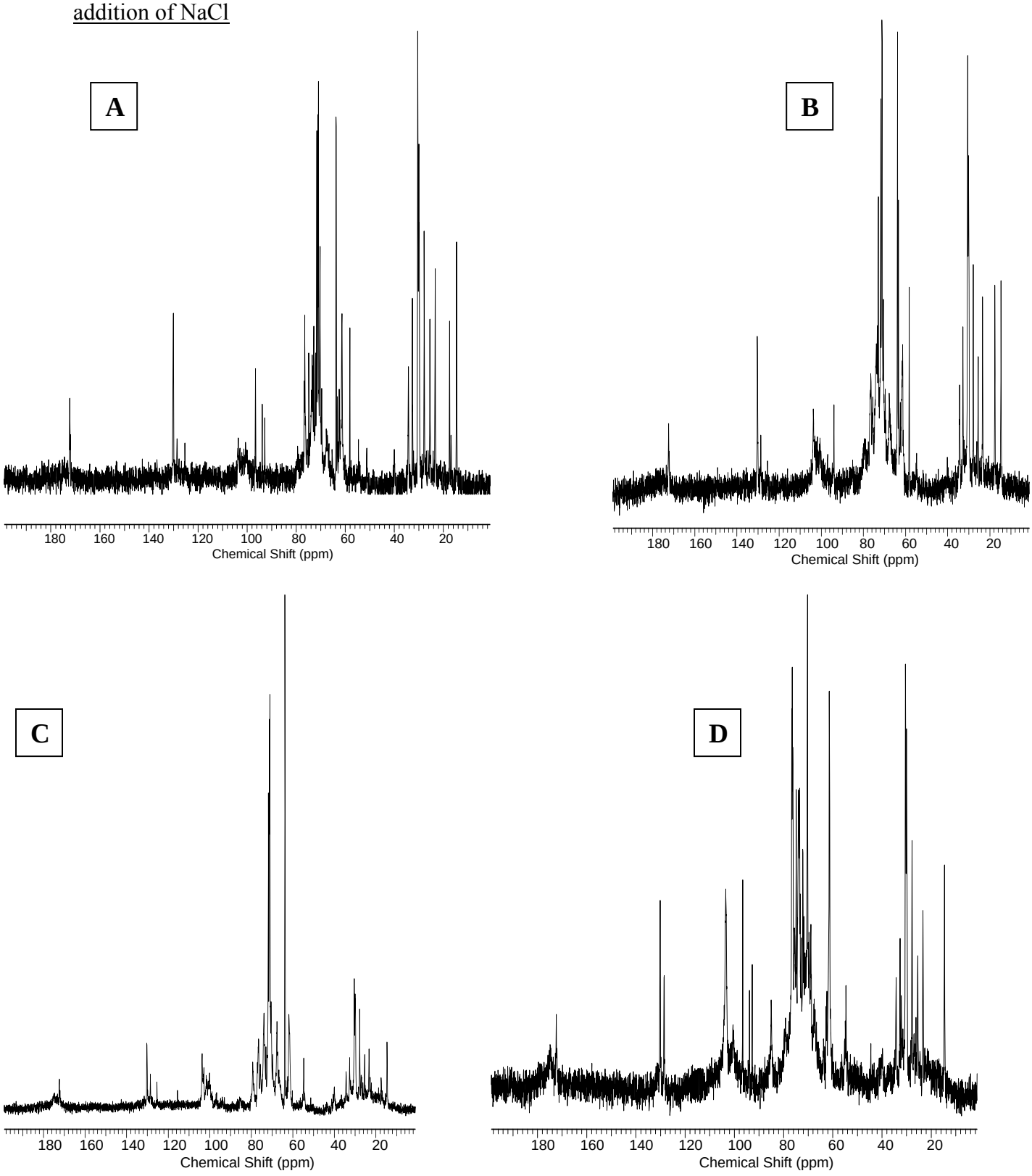

Figure 7.2.1: ^{13}C-NMR spectrum of *B. albus* CBS 6097 (A, B) and *B. dendrophila* CBS 6074T (C, D) when exposed to (A, C) 0.998 a_w, (B, D) 0.95 a_w (NaCl).

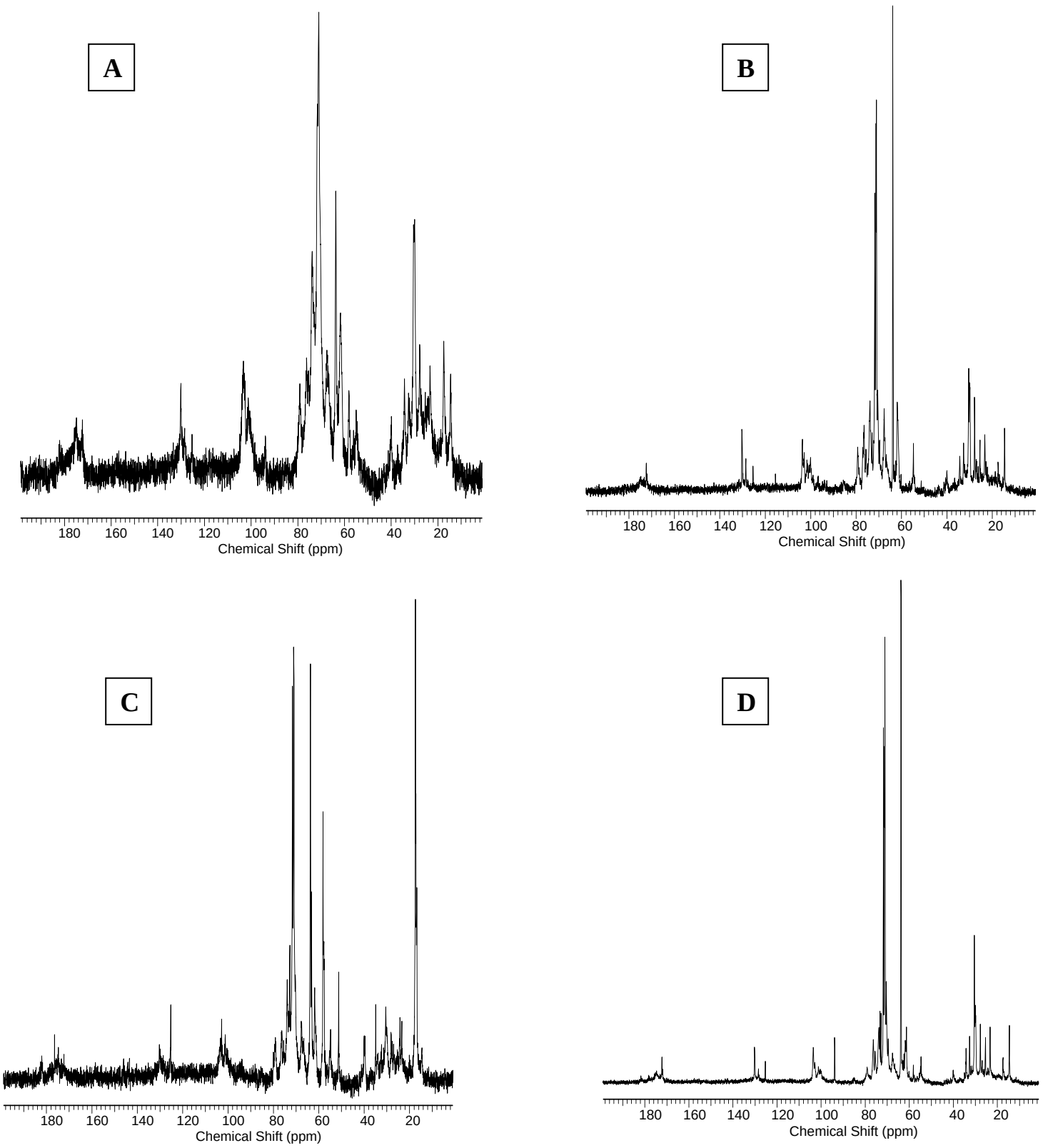

Figure 7.2.2: ^{13}C-NMR spectrum from organism, C. *albidus* CBS 5737 (A, B) and *C. amylolentus* CBS 6039T (C, D) when exposed to (A, C) 0.998 $a_{w,}$ (B, D) 0.95 a_w (NaCl).

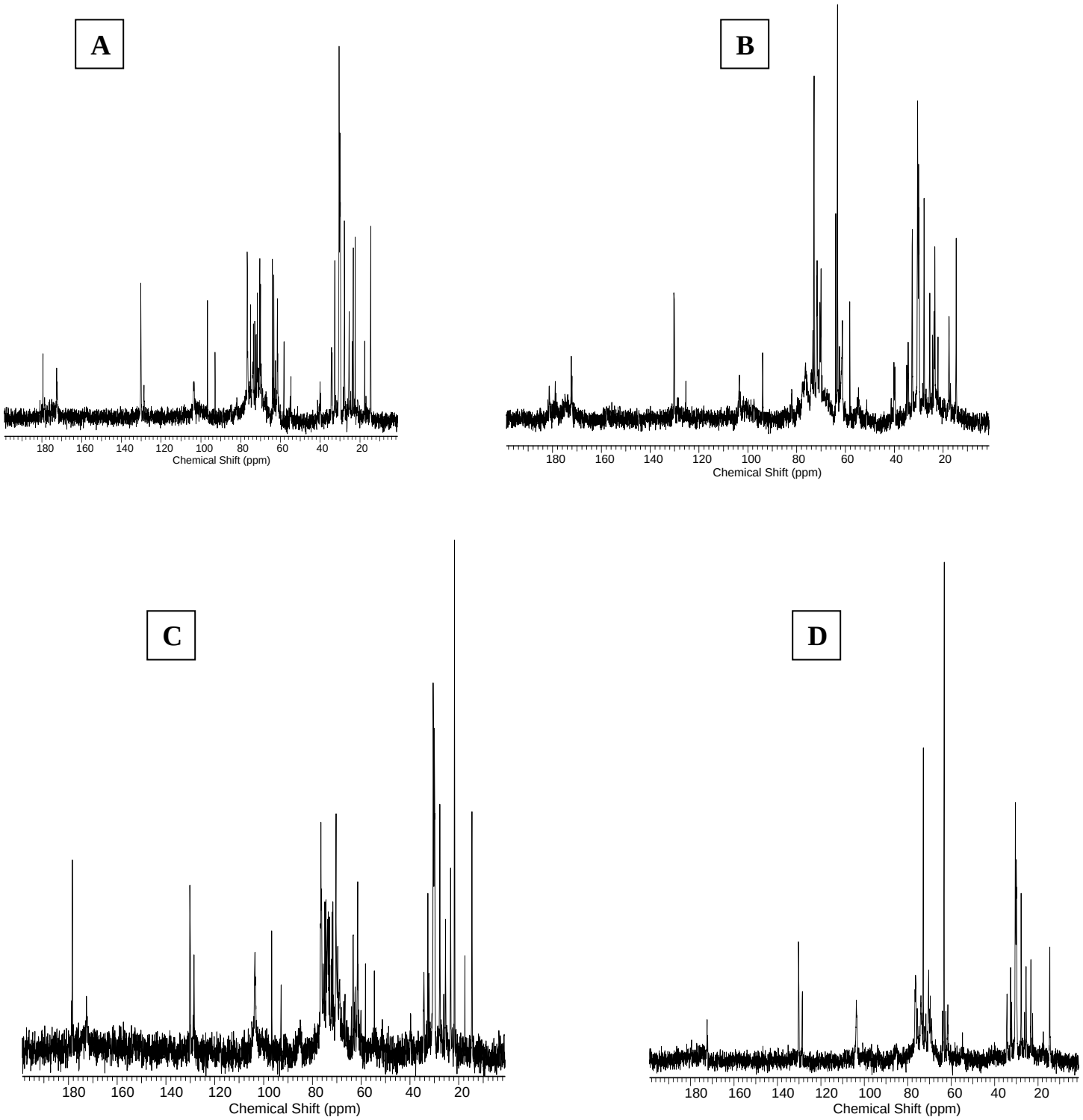

Figure 7.2.3: ^{13}C-NMR spectrum from organism, *C. bhutanensis* CBS 6294T (A, B) and *C. curvatus* CBS 2176 (C, D) when exposed to (A, C) 0.998 $a_{w,}$ (B, D) 0.95 a_{w}. (NaCl).

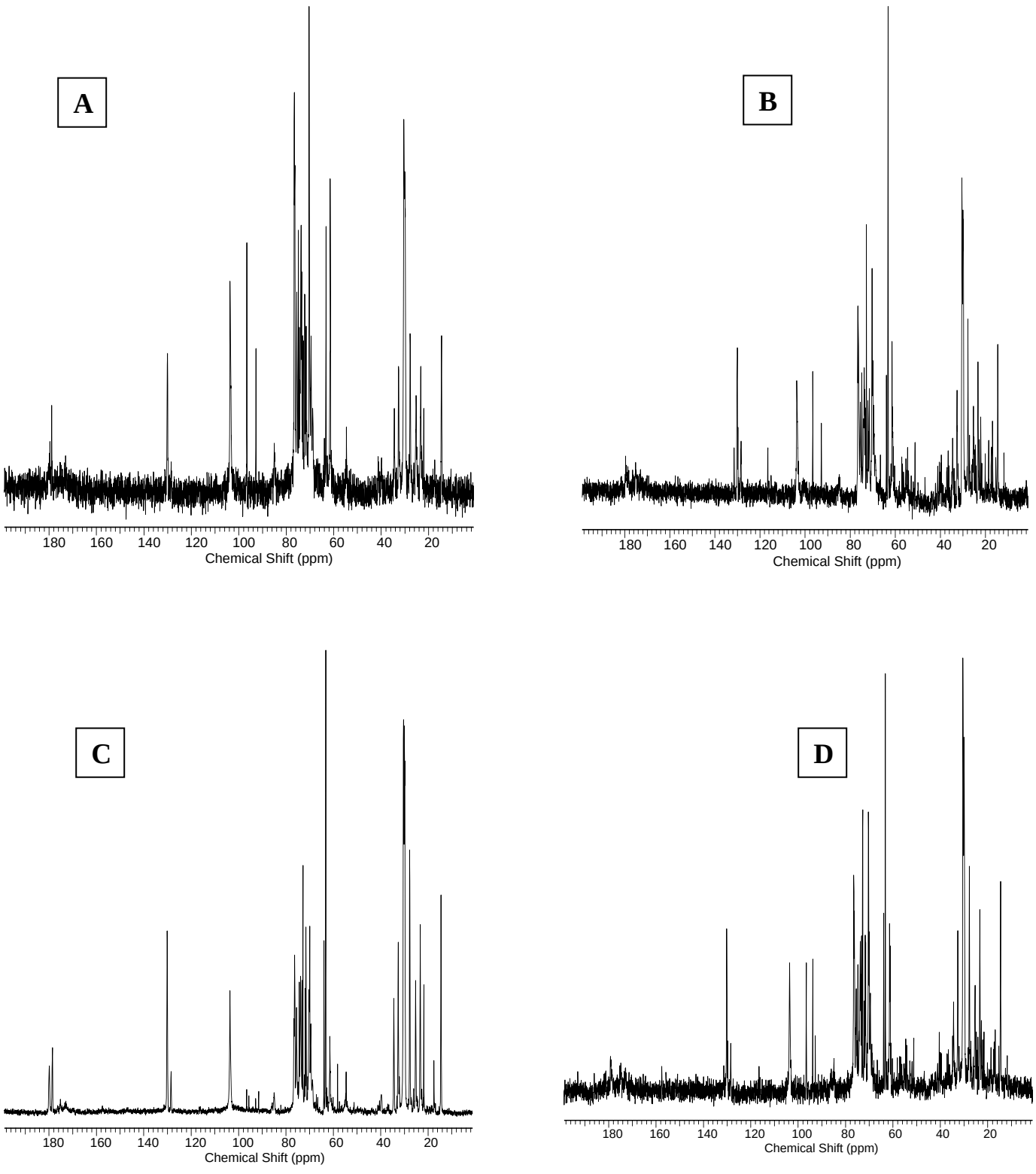

Figure 7.2.4: ^{13}C-NMR spectrum from organism, C. *laurentii* US 1A (A, B) and *C. laurentii* US 1F (C, D) when exposed to (A, C) 0.998 $a_{w,}$ (B, D) 0.95 a_w. (NaCl).

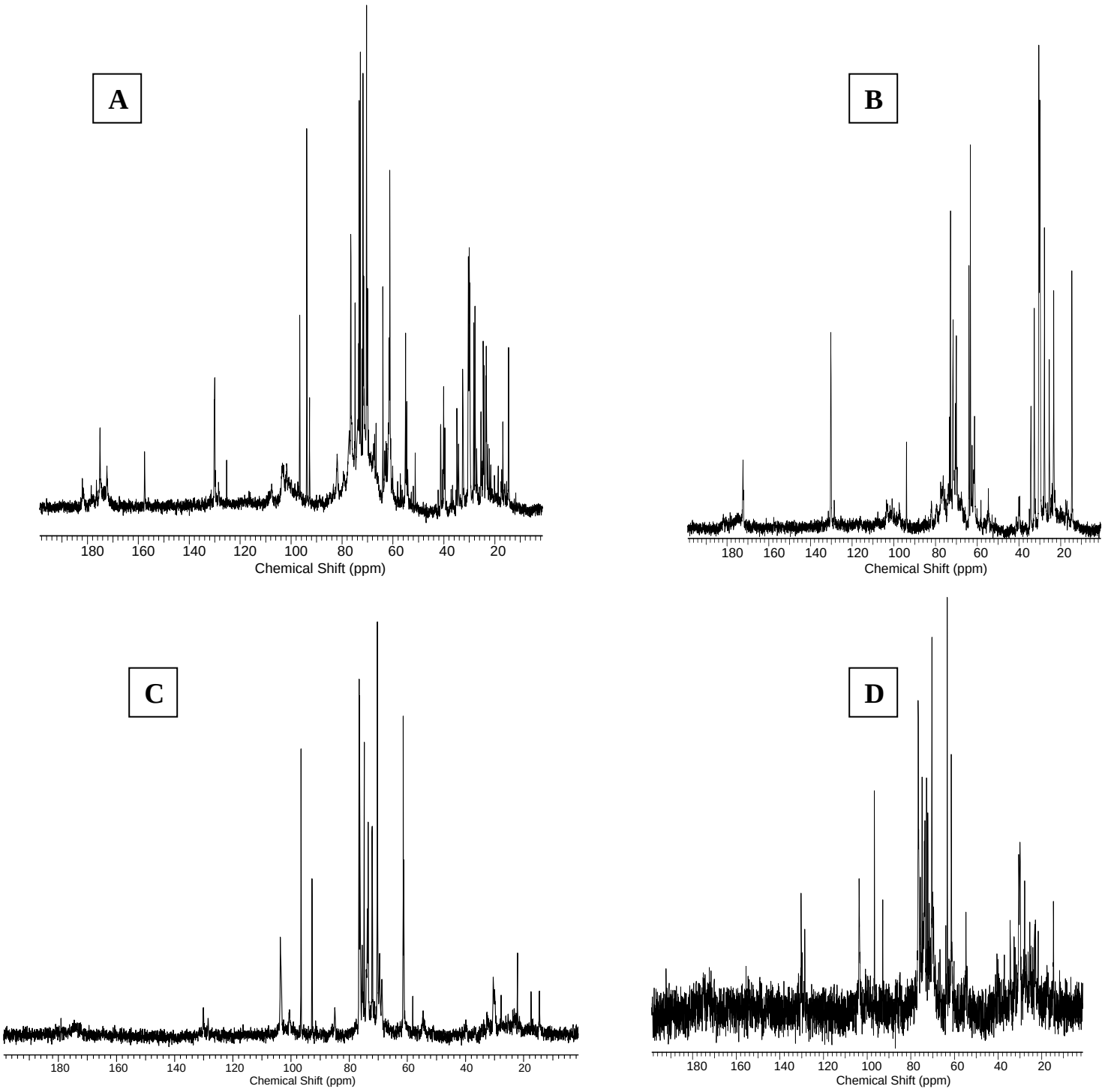

Figure 7.2.5: ^{13}C-NMR spectrum from organism, *C. macerans* CBS 2206T (A, B) and *C. neoformans* US 132T (C, D) when exposed to (A, C) 0.998 $a_{w,}$ (B, D) 0.95 a_w (NaCl).

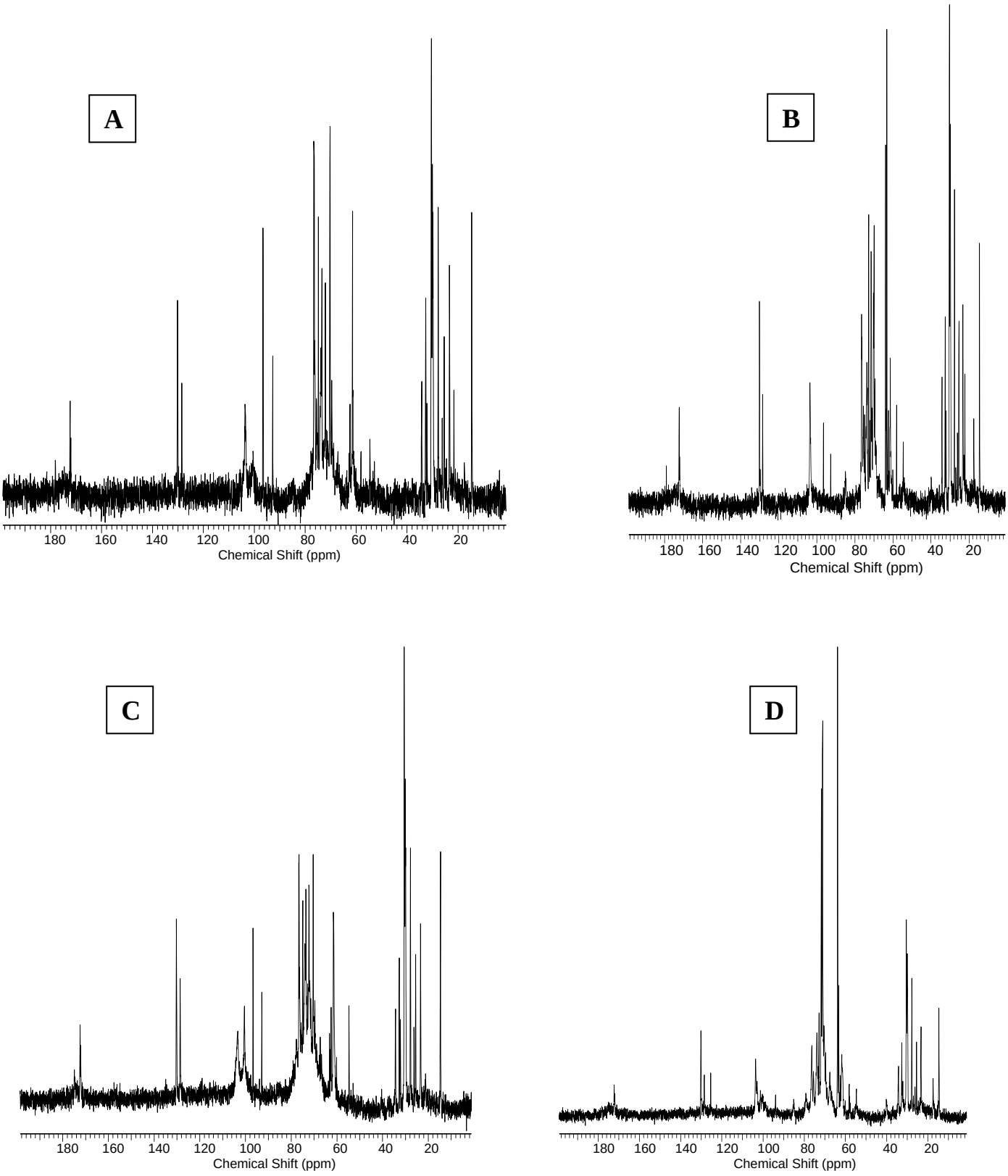

Figure 7.2.6: ^{13}C-NMR spectrum from organism, C. *neoformans* US C2 and *C. neoformans* US I1(C, D) when exposed to (A, C) 0.998 $a_{w,}$ (B, D) 0.95 a_w (NaCl).

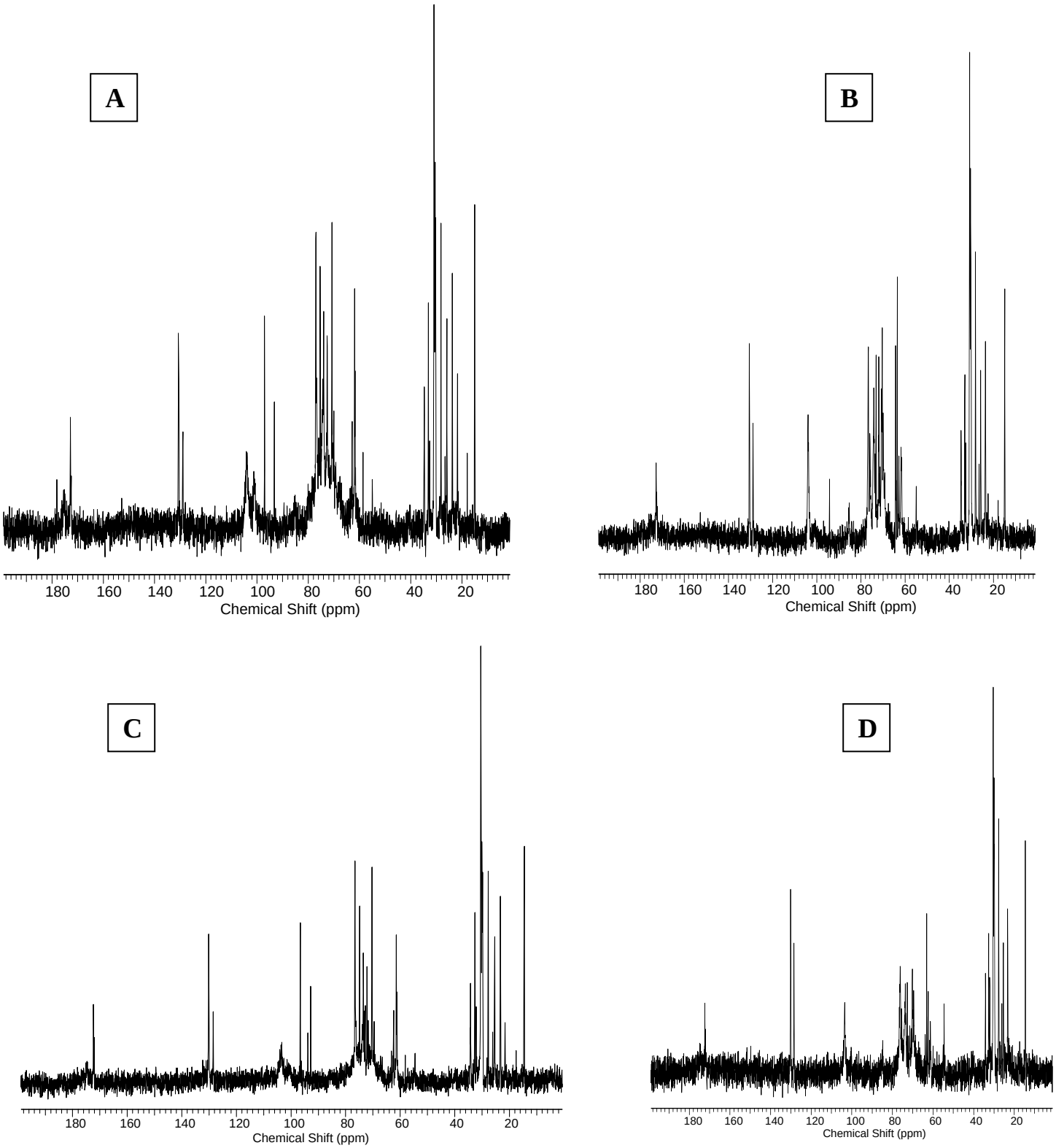

Figure 7.2.7 ^{13}C-NMR spectrum from organism, C. *neoformans* US I5 (A, B) and *C. neoformans* US I6 (C, D) when exposed to (A, C) 0.998 $a_{w,}$ (B, D) 0.95 a_w (NaCl).

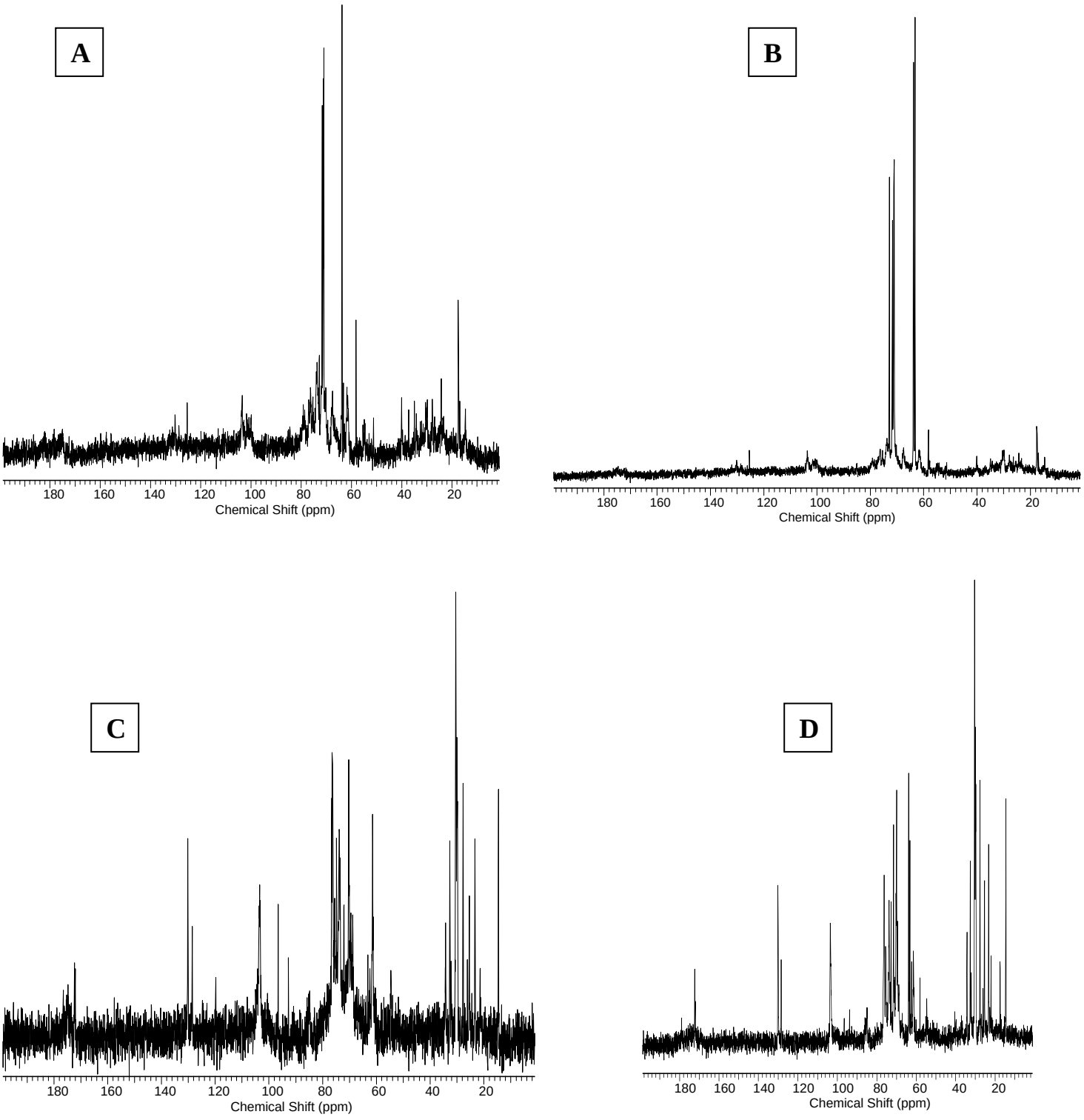

Figure 7.2.8 ^{13}C-NMR spectrum from organism, C. *neoformans* US S5 (A, B) and *C. neoformans* US I4 (C, D) when exposed to (A, C) 0.998 a_w, (B, D) 0.95 a_w (NaCl).

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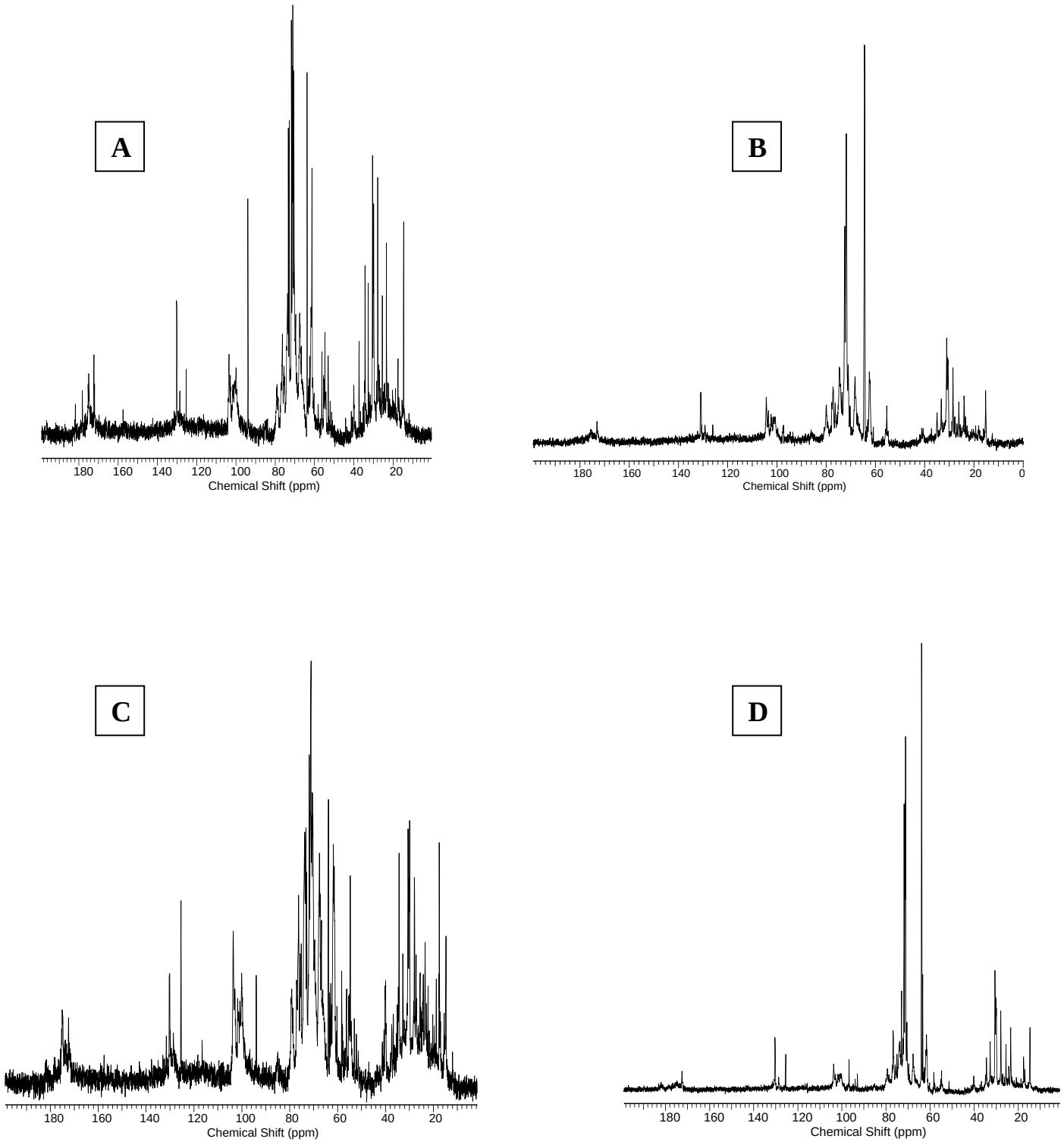

Figure 7.2.9: ^{13}C-NMR spectrum from organism, *C. podzolicus* US 5A (A, B) and *C. terreus* CBS 1895T (C, D) when exposed to (A, C) 0.998 $a_{w,}$ (B, D) 0.95 a_w (NaCl).

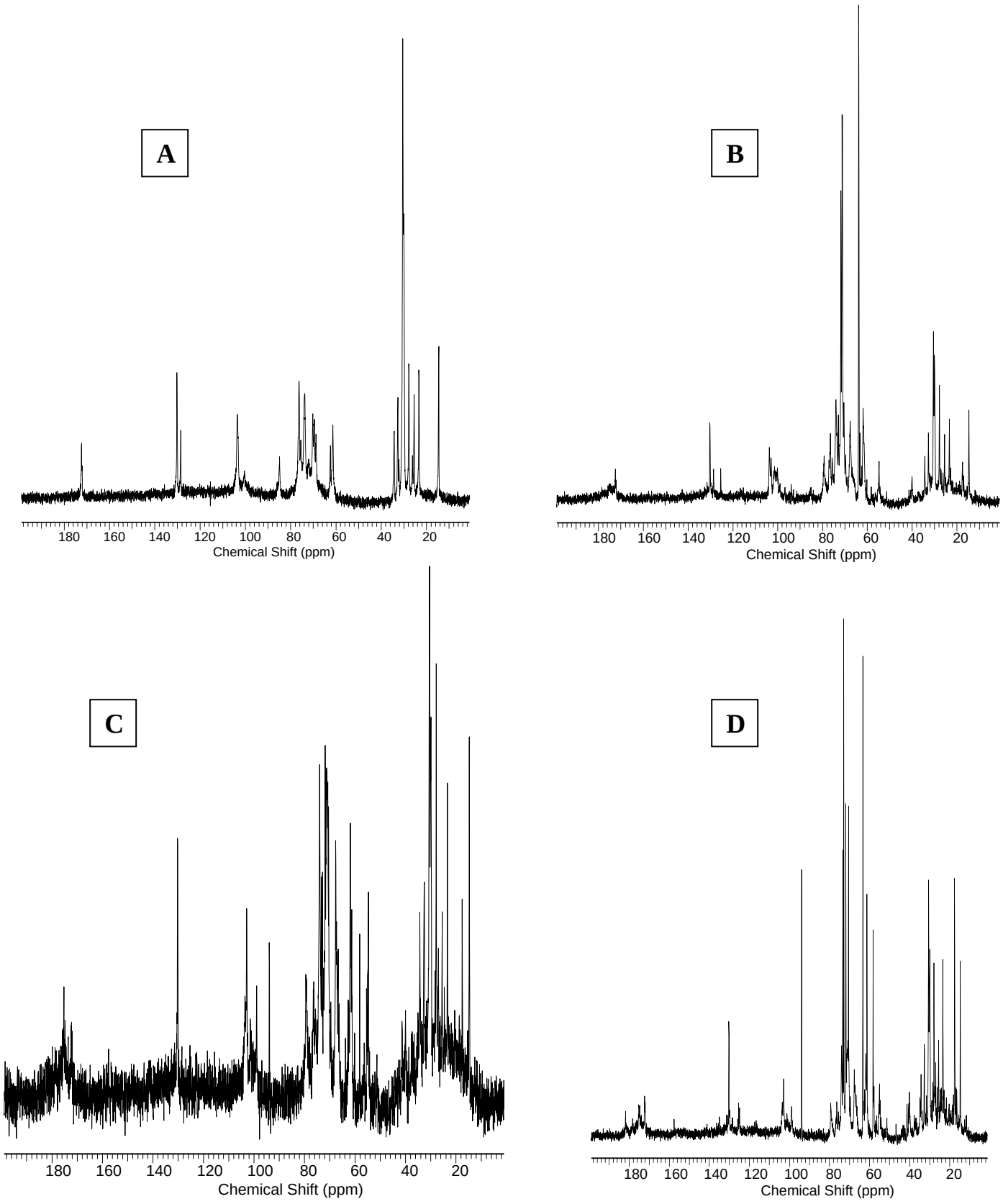

Figure 7.2.10: ^{13}C-NMR spectrum from organism, *C. laurentii* CBS 0139 (A, B) and *F. capsuligenum* CBS 4381 (C, D) when exposed to (A, C) 0.998 $a_{w,}$ (B, D) 0.95 a_w of NaCl.

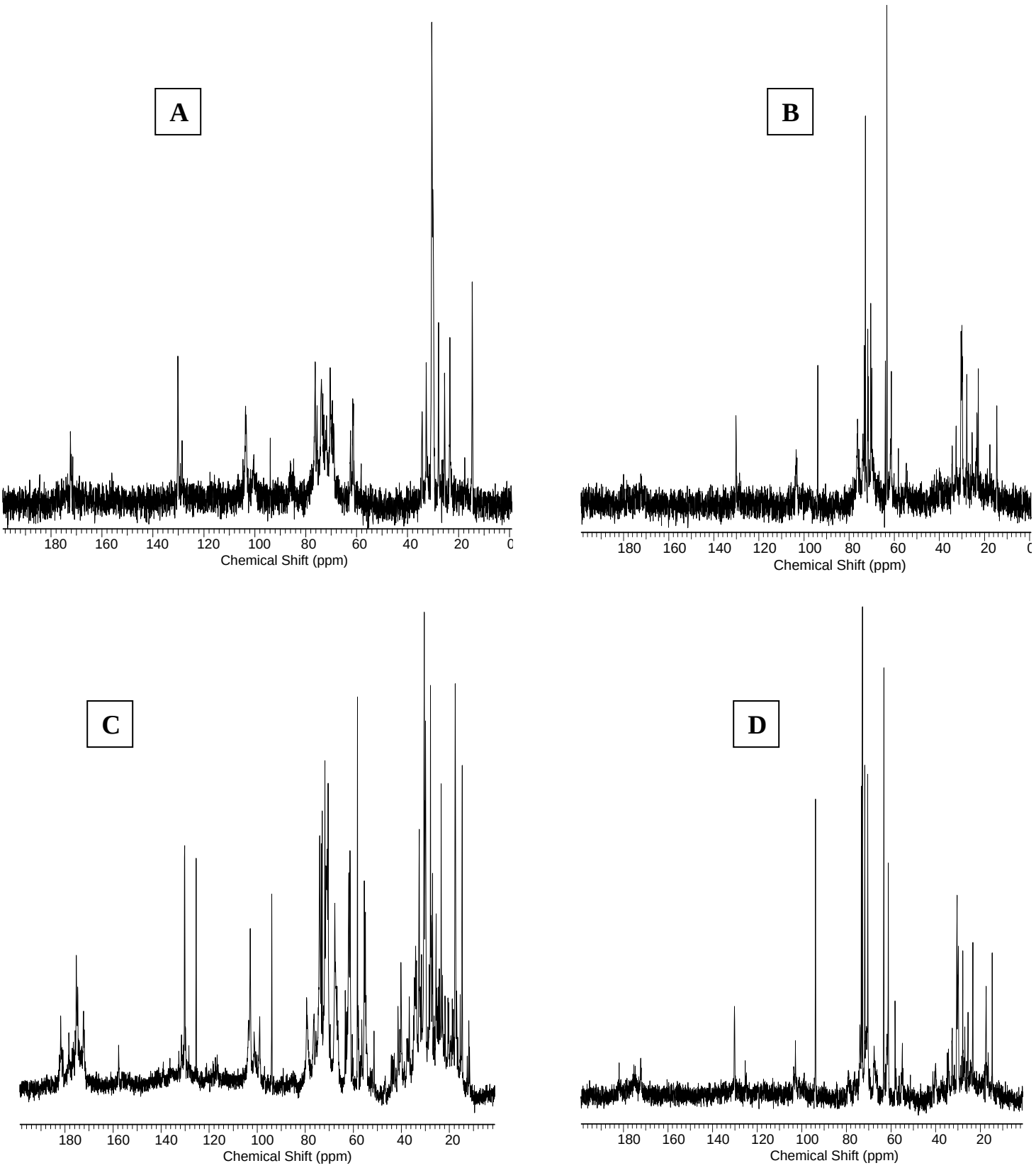

Figure 7.2.11: ^{13}C-NMR spectrum from organism, *F. capsuligenum* CBS 6122.2(A, B) and *F. floriforme* CBS 6240(C, D) when exposed to (A, C) 0.998 $a_{w,}$ (B, D) 0.95 a_w of (NaCl).

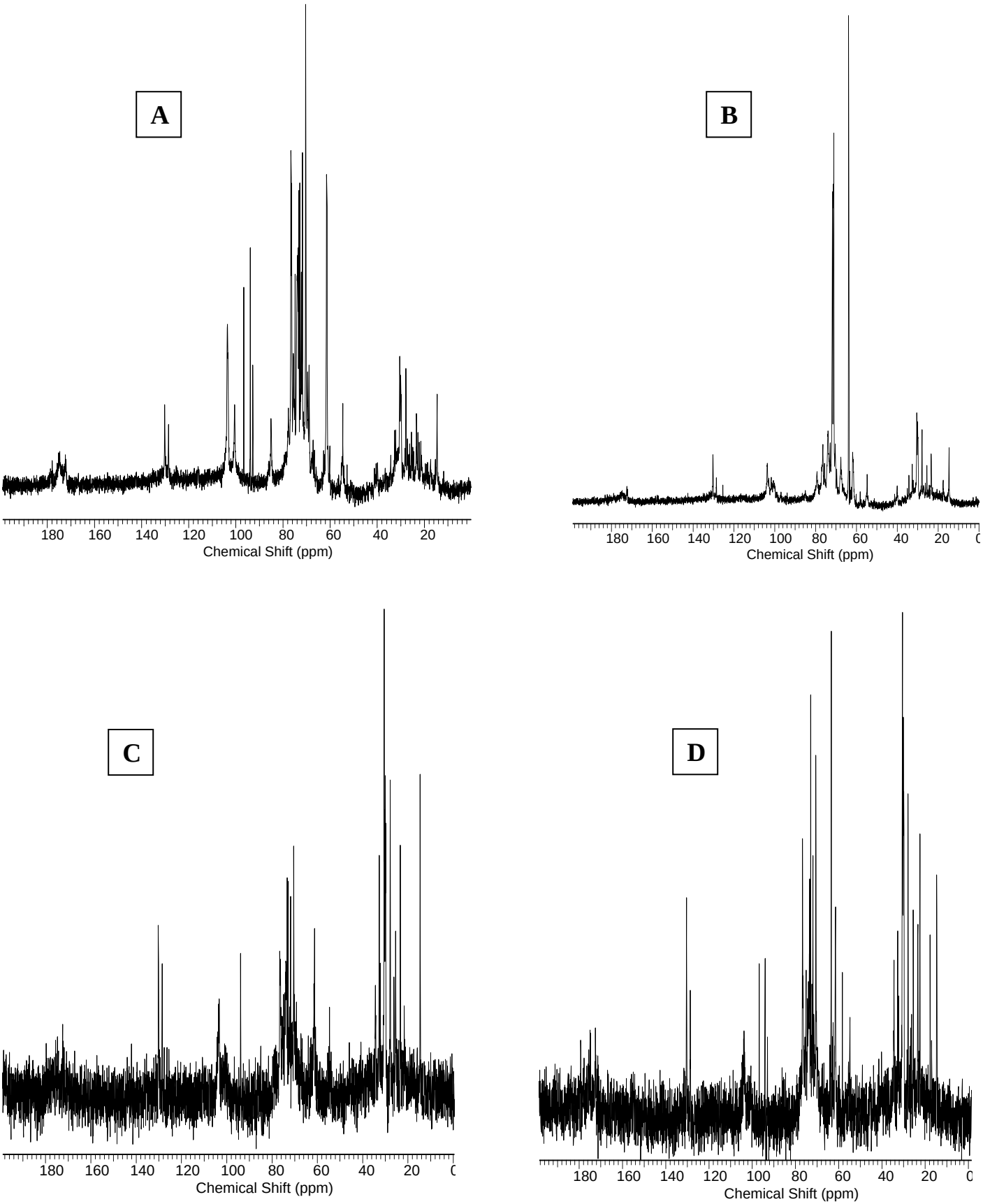

Figure 7.2.12: ^{13}C-NMR spectrum from organism, *F. neoformans* CBS 0132 (A, B) and *F. neoformans* CBS 0884 (C, D) when exposed to (A, C) 0.998 $a_{w,}$ (B, D) 0.95 a_w of (NaCl).

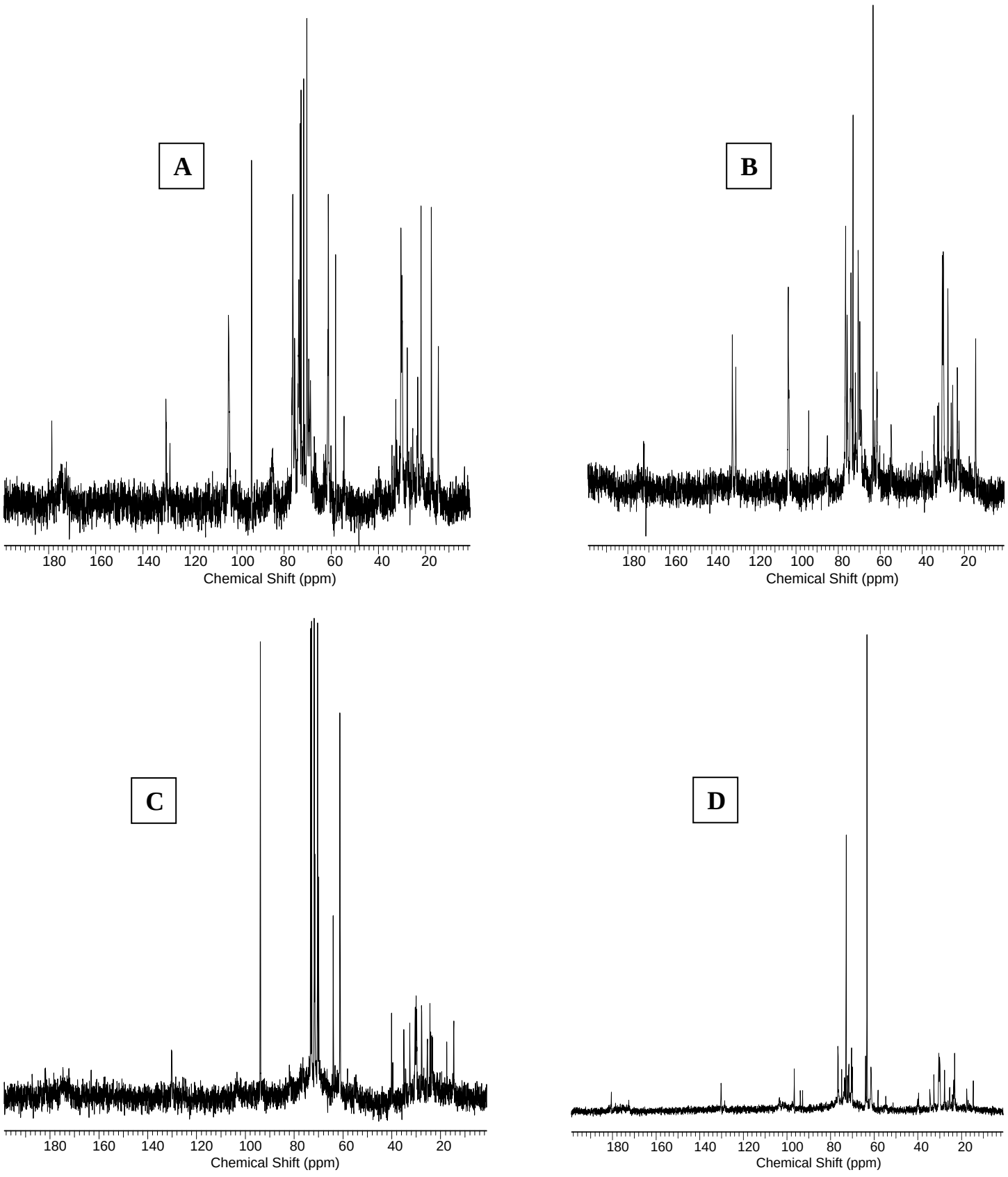

Figure 7.2.13: ^{13}C-NMR spectrum from organism, *F. neoformans* CBS 6885 (A, B) and *F. unigetulatum* CBS 2770 (C, D) when exposed to (A, C) 0.998 $a_{w,}$ (B, D) 0.95 a_w of (NaCl).

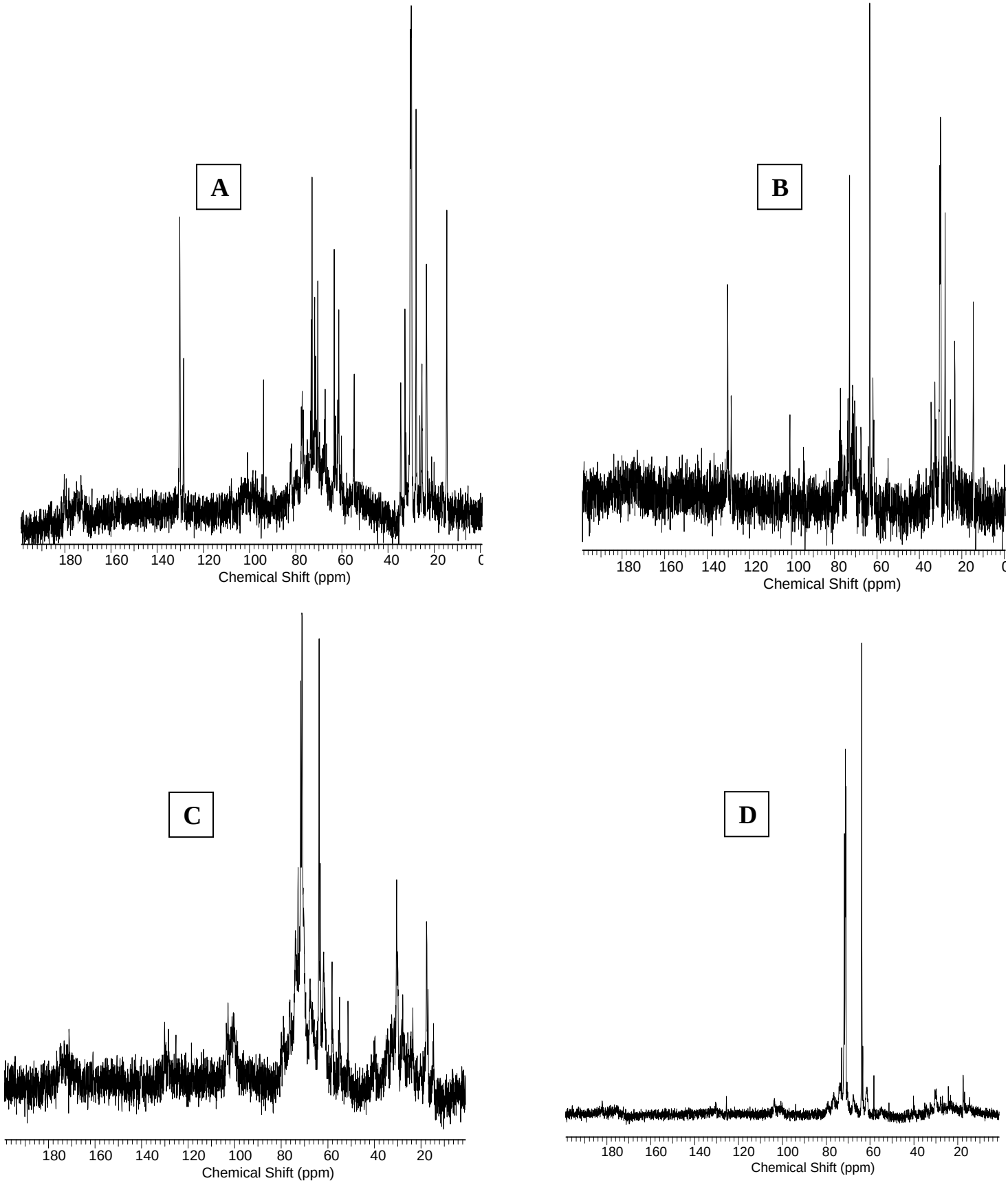

Figure 7.2.14: ^{13}C-NMR spectrum from organism, *R. araucariae* CBS 6031T (A, B) and *R. glutinis* CBS 0020 (C, D) when exposed to (A, C) 0.998 $a_{w,}$ (B, D) 0.95 a_w of (NaCl).

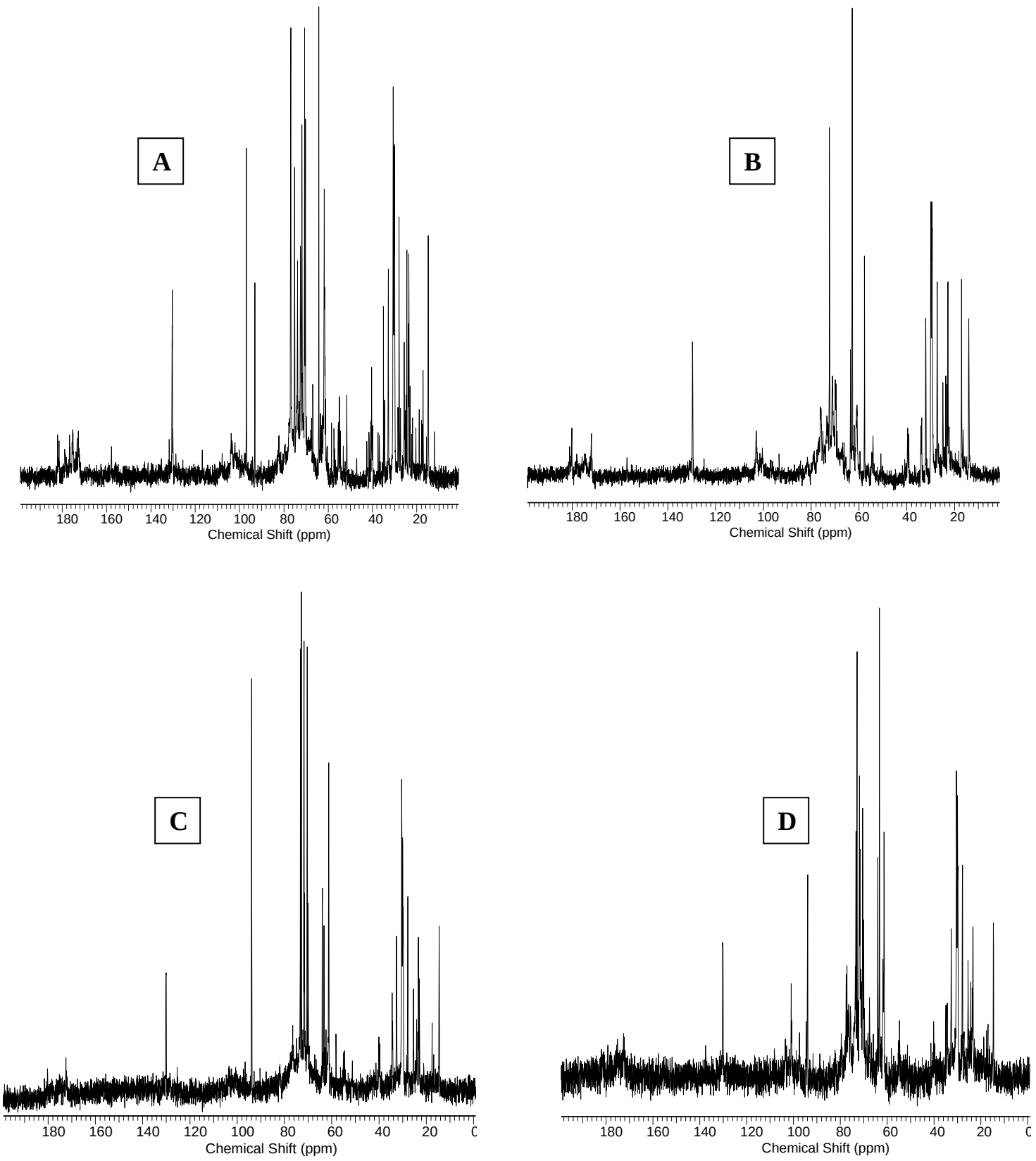

Figure 7.2.15: ^{13}C-NMR spectrum from organism, *R. graminis* CBS 2826 (A, B) and *R. lactosa* CBS 5826T (C, D) when exposed (A, C) 0.998 $a_{w,}$ (B, D) 0.95 a_w of (NaCl).

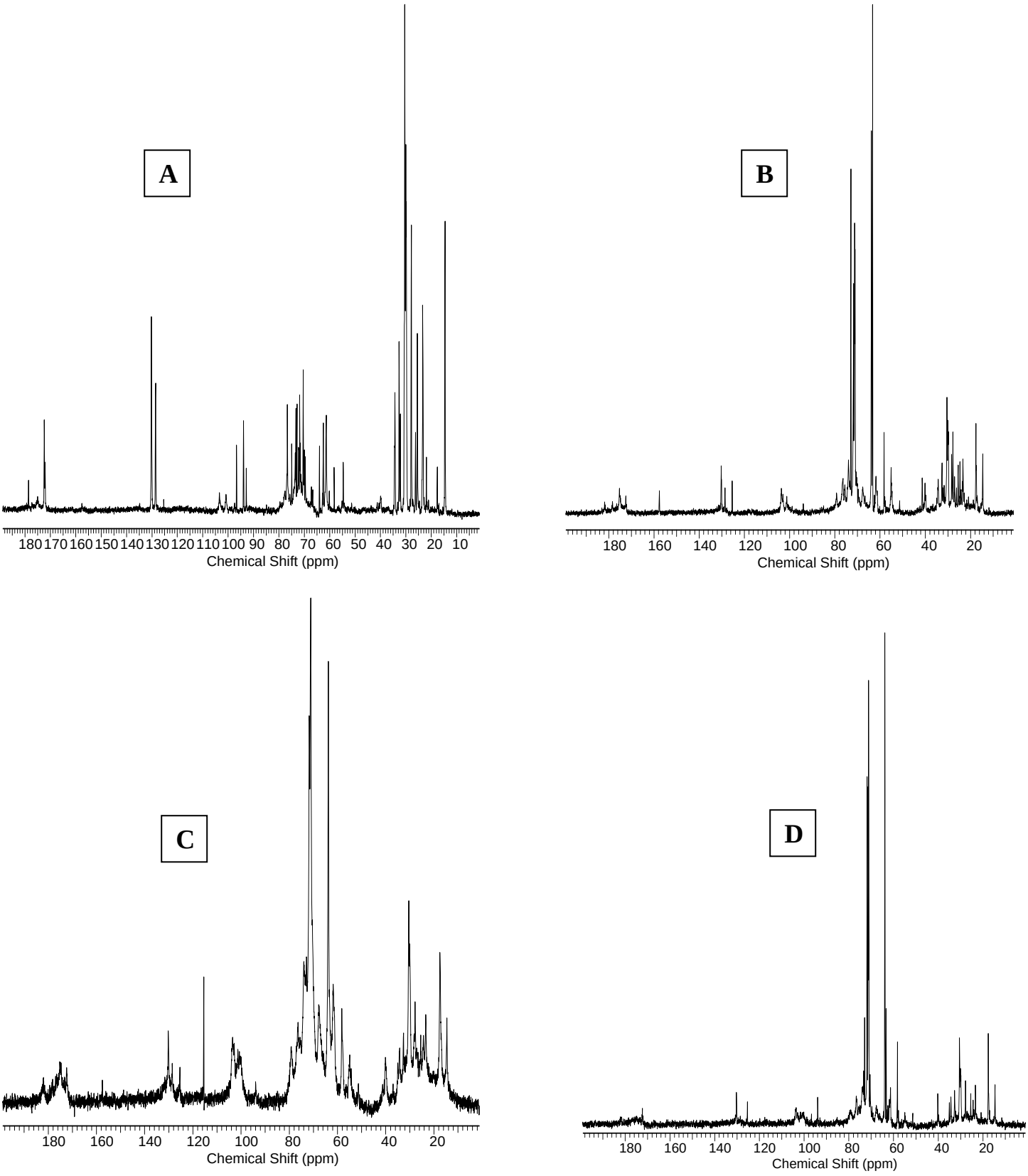

Figure 7.2.16: ^{13}C-NMR spectrum from organism, *R. minuta* CBS 2172 (A, B) and *R. minuta* CBS 2177 (C, D) when exposed to (A, C) 0.998 $a_{w,}$ (B, D) 0.95 a_w of (NaCl).

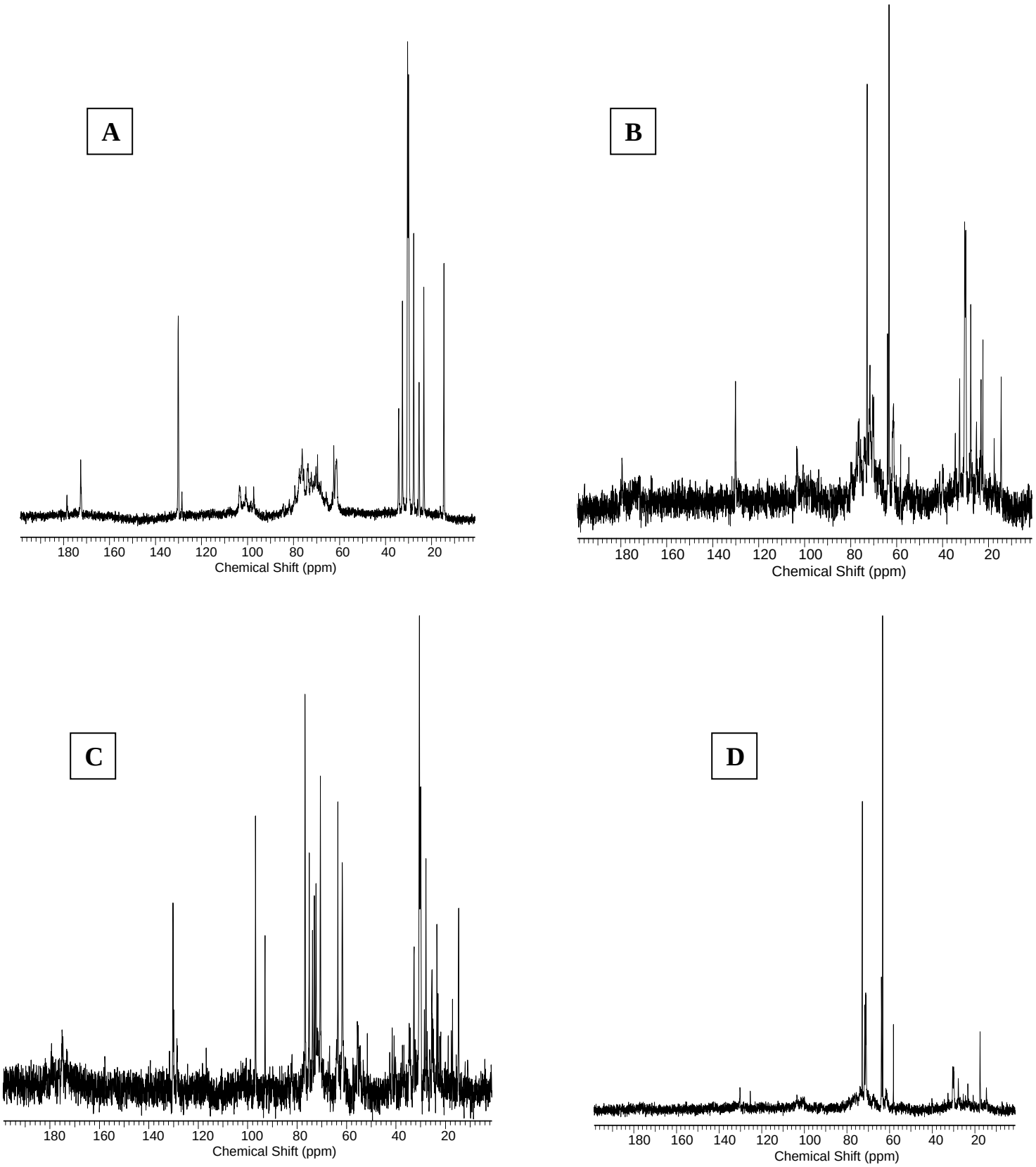

Figure 7.2.17: ^{13}C-NMR spectrum from organism, *R. mucilaginosa* CBS 5951 (A, B) and *R. toruloides* CBS 0349 (C, D) when exposed to (A, C) 0.998 $a_{w,}$ (B, D) 0.95 a_w of (NaCl).

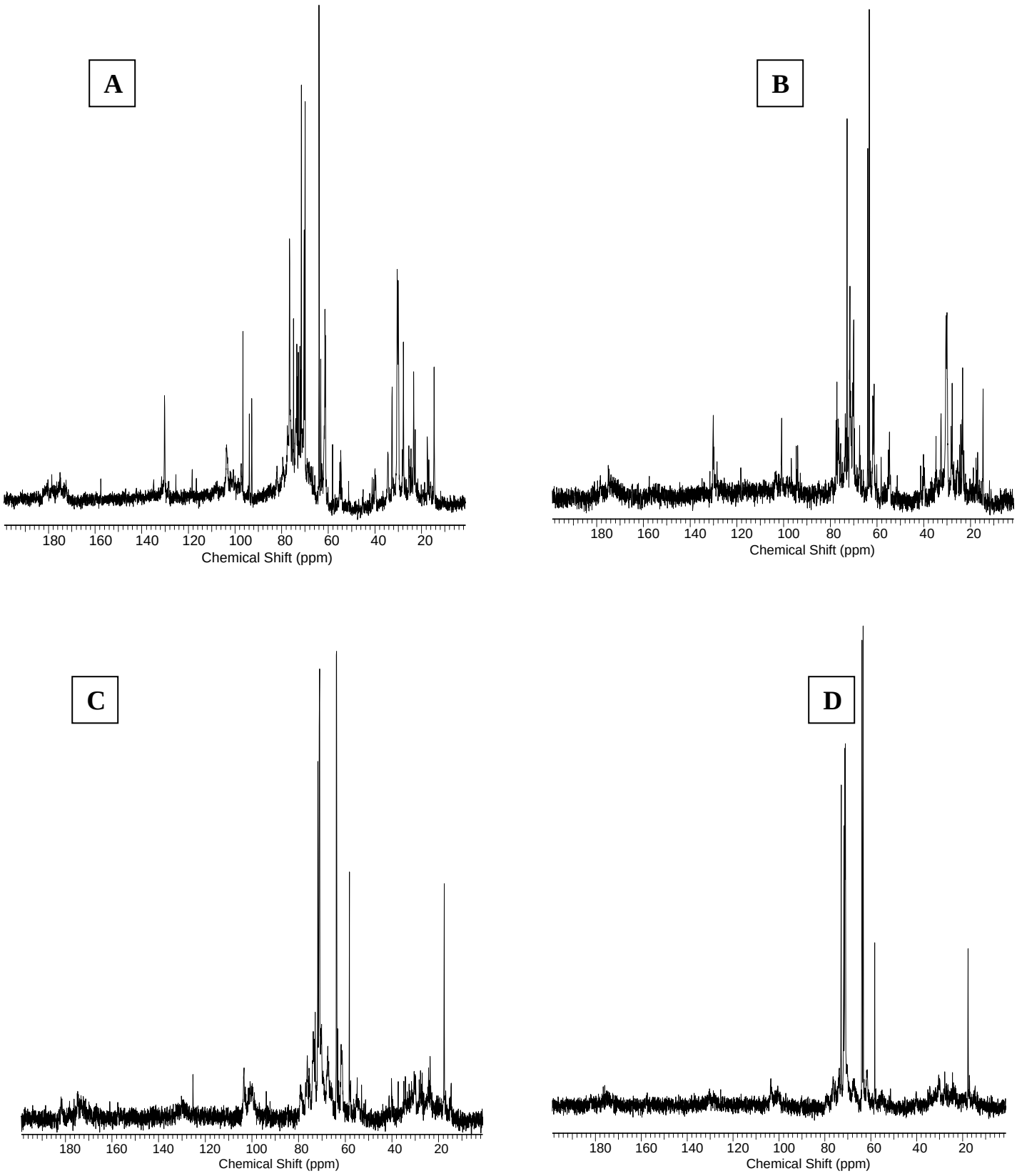

Figure 7.2.18: ^{13}C-NMR spectrum from organism, *Rhodotorula sp* CBS 5143 (A, B) and *T. cutenum* CBS 2466NT(C, D) when exposed to (A, C) 0.998 $a_{w,}$ (B, D) 0.95 a_w of (NaCl).

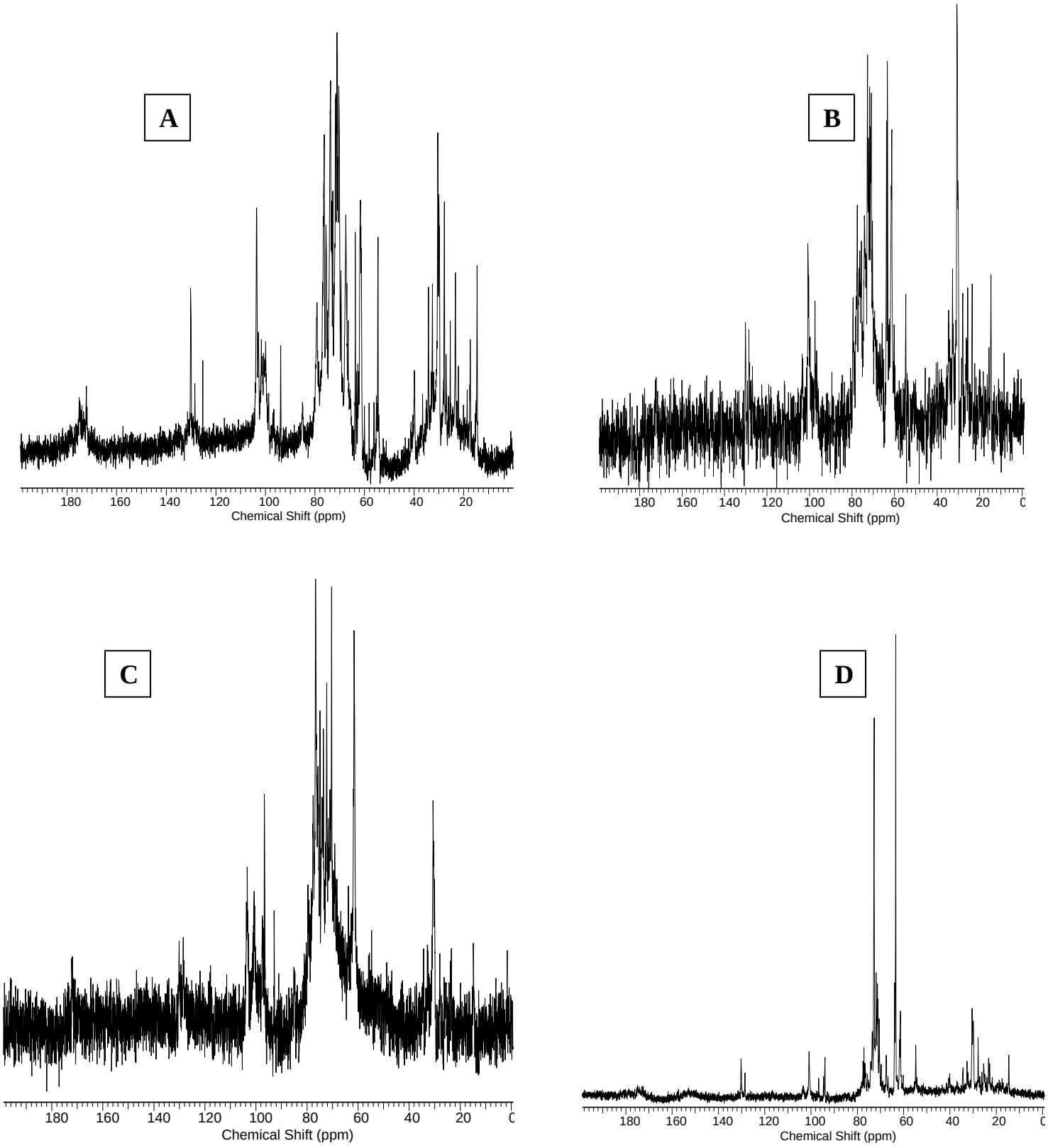

Figure 7.2.19: ^{13}C-NMR spectrum from organism, *S. halophilus* CBS 4609Y (A, B) and *S. halophilus* CBS 5628 (C, D) when exposed to (A, C) 0.998 $a_{w,}$ (B, D) 0.95 a_w of (NaCl).